Intermediate synthetic scientific report

2015

Intermediate scientific report encompass all activities developed during the first (year 2013), second (year 2014) and third part (2015) of the project.

During phase I of the project entitled “A new anti-invasive experimental strategy for infiltrative malignant gliomas”, project code PN-II-RU-TE-2012-3-0235, the following objectives, proposed in the realization plan, were achieved:

1. The evaluation of the genes involved in invasion of gliomas in the glioblastoma samples and in the primary glioblastoma culture, through qPCR analysis.
2. Evaluation of the genes involved in glioma invasion in the glioblastoma lines through qPCR
3. Development of a new experimental glioblastoma invasion model: model type “organotypic brain slices”

In the development of these objectives, the following activities took place:

A. Harvesting the glioblastoma samples through classical surgical procedure, “ open surgery” (Activ. 1.1) and stereotactic biopsy procedure (Activ. 3.1)
B. Obtaining the primary glioblastoma cultures and purchasing the glioblastoma lines (Activ.1.2 and Activ.2.1)
C. ARN extraction and deposit in a biological bank (Activ.1.3 and Activ.2.2)
D. qPCR analysis of the genes involved in glioblastoma invasion (Activ.1.4 and Activ.2.3)
E. Tissular sample extraction and processing, cultivation using the “organotypic brain slices” model and evaluation of the cell viability in the culture (Activ.3.2.)

A. Harvesting the tumor samples

Harvesting the glioblastoma samples used in this project has been accomplished through standard neurosurgical procedure (“open surgery “) or by stereotactic biopsy procedure. Tumoral fragments were obtained, extracted according to the standard surgical protocol, the tumor sample was selected from several fragments and directed towards microscopic analysis. Thus, tumor sample harvesting from this study did not influence the surgical or rezection grade, nor in the “open surgery”, and it did not prolong the stereotactic biopsy procedure time. Sample harvesting was conducted in full safety for the patient and consent for given both by the patient and the patient’s caregivers (Fig.1)

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Consimţământ informat pentru inrolarea in studiul:
„A new anti-invasive experimental strategy for infiltrative malignant gliomas"

Stimata doamna/Stimate domn,

Sunteţi invitat(ă) să participaţi într-un studiu despre modificările genetice în tumorile cerebrale. Scopul studiului nostru este de a identifica mecanismele moleculare implicate in invazia glioamelor cerebrale, pentru imbunatatirea diagnosticului si terapiei.
Participarea dumneavoastra este voluntara.

PROCEDURĂ: Veţi fi rugat(ă) să oferiţi o probă de tesut tumoral in timpul operatiei.
In timpul operatiei, o mica portiune din tesutul operat va fi prelevat in vederea extractiei de acizi nucleici si proteine pentru identificarea unor markeri specifici tumorilor cerebrale.
Dorim ca după încheierea studiului de faţă să păstrăm eventualul rest din proba ADN/ARN/proteica obţinută. Aceasta va fi congelată şi depozitată sub un cod şi nu direct cu numele dumneavoastră.
În cazul în care doriţi, vă puteţi retrage oricând din studiu, fără ca aceasta să afecteze în vreun fel dreptul dumneavoastră la tratament, inclusiv după ce aţi semnat acest formular.
Rezultatele studiului pe proba dumneavoastră sunt confidenţiale şi vor fi folosite numai în scop de cercetare.

BENEFICII ale participării:
- Ajuta la imbunatatirea protocolului de diagnostic
- Ajuta la dezvoltarea de noi terapii

RISCURI:
- Nu sunt riscuri suplimentare fata de cele asumate in consimtamantul operator

PLĂŢI ŞI ALTE BENEFICII:
- Nu veţi beneficia terapeutic din acest studiu, deoarece în acesta nu se administrează niciun medicament
- Nu veţi fi plătit(ă) ca să participaţi la acest studiu

CONFIDENŢIALITATE ŞI STATUT
Doar cercetătorii implicati in proiect şi un reprezentant al Comitetului de Etică al Spitalului vor avea acces la datele adunate pe parcursul acestui studiu. Utilizarea unor informaţii de tip personal este securizată conform legislaţiei în vigoare.
Deoarece informaţiile despre dumneavoastră şi despre starea dumneavoastră de sănătate sunt personale şi private, ele nu pot fi folosite în scop de cercetare fără acordul scris al dumneavoastră. Semnând acest formular, ne veţi da acordul dumneavoastră în acest sens.
Acest formular are scopul de a vă informa asupra felului în care datele despre sănătatea dumneavoastră vor fi folosite în acest studiu. Vă rugăm să citiţi cu atenţie înainte să semnaţi.

DATE DE CONTACT

Dacă aveţi întrebări legate de studiu sau dacă apar probleme, puteţi contacta persoana responsabila de studiu:

Dr. Felix Mircea Brehar, Spitalul Clinic de Urgenta „Bagdasar-Arseni", cu sediul în Bucuresti, cod poştal 041915, str. Soseaua Berceni nr. 10-12, judeţ (sector) 4, tel. 0213343025/int. 1707, mobil: 0724257549, fax 0213347350.

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Declaraţia pacientului

(Incercuiti raspunsul corect)

Sunt / nu sunt de acord ca probele mele biologice să fie folosite in cercetări ulterioare

Sunt / Nu sunt de acord cu testul/testele descrise în acest formular.

- Sunt de acord ca probele sa fi depozitate în Laboratorul de Cercetare al spitalului pentru uz ulterior.
- Înţeleg faptul că proba ar putea fi trimisă spre un alt laborator în afara Spitalului Clinic de Urgenta „Bagdasar-Arseni".

Semnătura pacientului .... Data..............................

Nume (Litere de tipar) ....CNP.................................

Semnătură apartinator ruda gradul I.. Dată ....

Nume (Litere de tipar) ....

Note importante: (bifaţi dacă este cazul)
- **Pacientul şi-a retras consimţământul (rugaţi pacientul să semneze aici) ....**
Data:........................

Declaraţia personalului medical

Am explicat procedura pacientului. În mod particular i-am explicat beneficiile şi riscurile aşa cum apar în acest formular.
Am discutat de asemenea ce ar putea implica procedura, beneficiile şi riscurile oricărei testări alternative (inclusiv lipsa unei testări) şi orice problemă care preocupă pacientul.

Semnătura:.. Data ....

Nume (Litere de tipar) ....

Statut (investigator / medic curant) ....

Fig.1 – Consent form model, filled in by the patient and the guardian in the present study

Grade II,II,III cerebral glioma (glioblastoma) patients were included in the present study, diagnosis was confirmed by the hisotpathological examination (parafined samples colored with HE) ± imunohistochemical analysis. The reason why grade I cerebral gliomas were not included in this study is the fact that this type of tumors have distinctive histopathological particularities from the other glade gliomas (like the policytic astrocytoma) moreover, they are circumscribed and they do not have a tendency towards invasion (1,2).

a. Sample harvesting through classical neurosurgical "open surgery"

The neurosurgical technique of tumor removal was selected for good delimited tumors, localized in the ineloquent cerebral areas, accessible only through surgery and with an important mass effect on the adjacent cerebral structures (Fig.2), at which the initial plan was mass reduction at which we could anticipate a tumor resection as large as it could be without any major risk of postoperative neurological deficits.

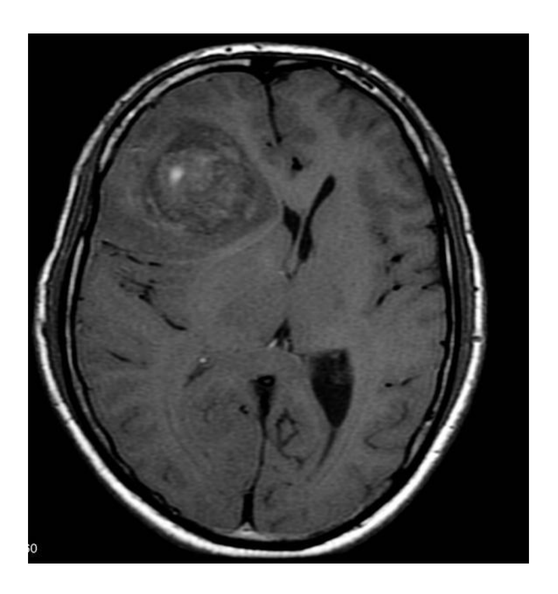


Fig.2. Right frontal glioblastoma (grade IV glioma). The tumor has a relatively good delimitations, localized in the right frontal hemisphere (non-dominant hemisphere) with mass effect. Patient has indications for "open surgery".

Steps of the operations:

- Induction of anesthesia (general anesthesia with oro-tracheal intubation)
- Positioning and preparations of the operatory field
- Craniotomy
- Opening the dura mater and explosion of the tumor-infiltrated cerebral area that holds edema and modified vascular pattern
- Tumor removal
- Hemostasis
- Closing of the plans

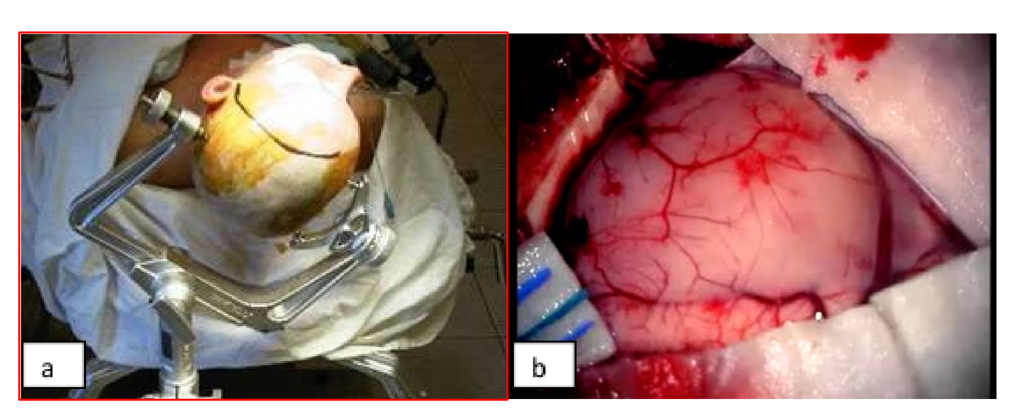


Fig. 3 Operatory steps of the neurosurgical intervention "open surgery"

B.Sample harvesting through stereotactic biopsy

Patients selected for stereotactic biopsy presented infiltrative cerebral gliomas (Fig. 4a), localized in profound or eloquent cerebral areas (Fig. 4b), at which tumor exertion could not be accomplished without a major risk of postop neurological deficits.

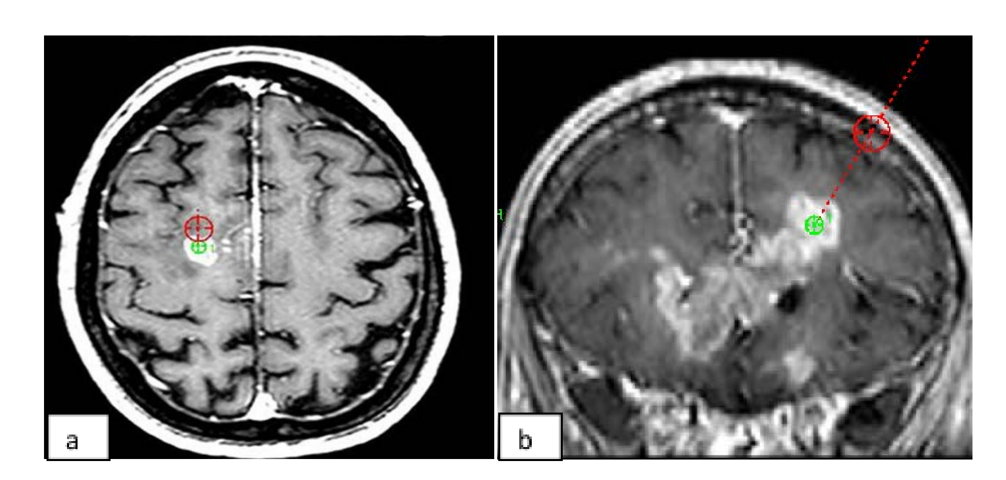


Fig.4 Types of cerebral gliomas selected for the stereotactic biopsy (T1 MRI with snapshot contrast, selected during the preoperator time) – Sp. CL. “Bagdasar-Arseni”

All stereotactic procedures were done by Dr. Felix Brehar, using the Leksell stereotactic system (Fig.5) and Stereotactic Planning System software (SPS), versiune NTPS 8.2 (Elekta, Sweden). For the scanning of the patient, type 1.5 MRI Tesla Magnetinc Resonance (Philips Integra). The system utilized by the author for the finalization of the procedure is one of the most error-free (medial error below 0.5mm and max below 1mm). The biopsy needle used was Type I Sedan (Elekta, Sweden) with a slot of 10mm. The steps of the stereotactic bypsy are :

-fixation of the stereotactic frame

-MRI or CT scan of the brain

-procedure planning

-stereotactic biopsy (fig.6)

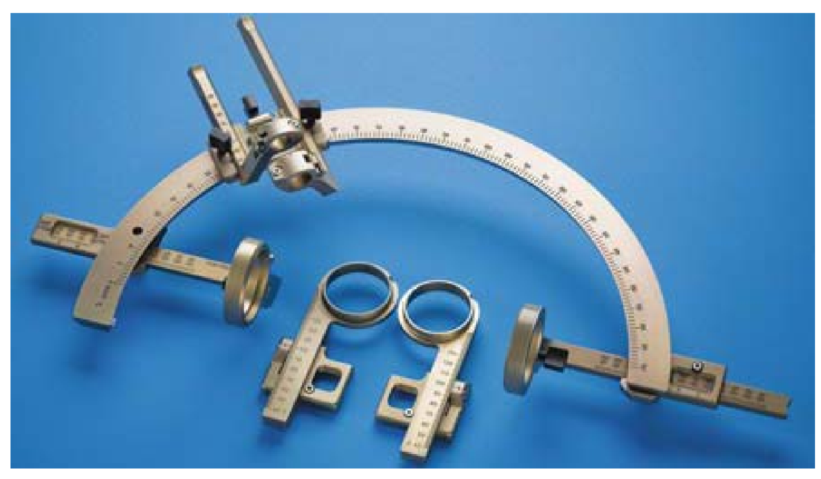

Fig. 5. Leksell stereotactic system



Fig. 6. Intraoperatory snapshot during the procedure of the stereotactic cerebral biopsy – Sp. Cl. “Bagdasar-Arseni”

Intraoperative tumor samples selected for inclusion in the study had sizes less than 1 cm, were cleaned of blood and cellular debris and were included in a sterile conditions in eppendorf tube filled with 1.5 ml RNA solution saver and were immediately stored at 2-4 ° C 24 hours and then at -80 ° until RNA extraction.

By the time of reporting 30 patients were included in the study with brain gliomas in which from 15 patients tumor samples were collected by standard surgical technique "open surgery" and 15 were performed the stereotactic biopsy. In 11 patients in which the tumor was located in ineloquent areas in the frontal and temporal pole, standard resection procedure was performed for frontal and temporal pole. In these cases we have achieved complete tumor ablation and harvesting of the peritumoral cerebral tissue could be performed in safety conditions. These samples have been utilized as a reference. We have extracted RNA from 21 out of 30 cases, having a total of 31 probes by the time of the reporting (21 tumor and 10 peritumoral).

b.Obtaining the primary glioblastoma cultures and purchasing of the glioblastoma lines

In two cases with high-volume tumors (patient 15 and 16) at which the open neurosurgical intervention was performed, sampling of several femoral fragments was possible, from which primary glioblastoma cultures were initiated. It is very important to mention the time the fragments stayed in physiological serum or culture environment until processing. Processing of the tumoral fragments was performed in the same day, at a maximum of 2-3 hours from intraoperative harvesting. It the preservation time of the tumors surpasses several hours, it is expected that enzymatic extra and intracellular reactions should be induced, with cellular damage, modification of the tumor cells properties, viability decrease through membrane sensibilization at the action of enzymatic systems, the lowering of the adhesion rate post cryoconservation, until massive cellular destruction through osmotic lysis, enzymatic etc.

The processing of the fragments is done in perfect sterile conditions, at a laboratory hood.

Before mechanic processing, the tumor fragments were washed 3 times in physiological serum or PBS (phosphate buffer solution), for the removal of blood stains and cellular debris.

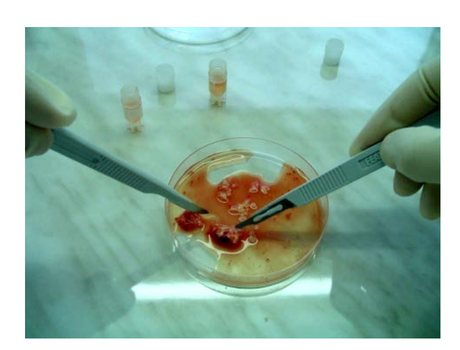


Fig. 7. Mechanical processing of the tumoral fragment in the laboratory hood

Processing of the eloquent cellular groups is done with soft instruments, extremely sharp, sterile, microsurgical instrumentary, in a Petri dish of medium dimensions. It is extremely important in microscopical recognition of the relevant tumoral parts, removal using a scalpel blade of the necrosis, coagulated portions, cerebral veins/arteries or neoformation tumor cells and their clots, fibrous zones from the tumor capsule, gliosis cerebral portions, normal peritumoral brain-tissue, fat tissue fragments, muscular, etc. The relevant tumor parts are of brown-grey-red color, and their recognition is possible only through gained experience, from multiple tumor exertions. After eliminating the irrelevant tumor parts, the viable tumor remainder is sectioned repeatedly with a scalpel, until milimetrical fragments ar obtained.

The steps of primary glioblastoma cultivations were :

1. Enzymatic dispersion and mechanical tissular fragmentation
2. Suspension in culture environment DMEM with 20% fetal serum
3. Seriate confluence subcultures 85-90%

The utilized environments were : DMEM (Dulbecco Modified Essential Medium) +3% Penicillin and Streptomycin +20% fetal serum, PBS(Phosphate buffered saline solution) 0,01M, Tripsine 1:250, 1% glucose.

Tissular dispersion proved to be more efficient and much faster when tripsine solution was used compared to EDTA, in exchange, cellular adhesion and monolayer formation occurred much slower with enzymatic dispersion. As a result, fragment dispersion through tripsine and EDTA, although slower, it protects cells and favorize adhesion and etalation.

The dispersion time is maintained at 2-3 minutes; the membrane integrity is damaged at over 5-8 minutes and the cells do not adhere. EDTA in optimum concentration 20mM act as a chelating agent of Ca+2 (Ca+2 is active in the intercellular adhesion). 1% Glucose of tripsine assures a lager percentage of viable cells if adequate osmolarity.

Also, experimentally we noticed that tripsine inactivation is more efficient if it is achieved by adding fetal serum as opposed to ice inactivation.

We have cultivated cells for 20 passages, observing the fenotipic aspect.

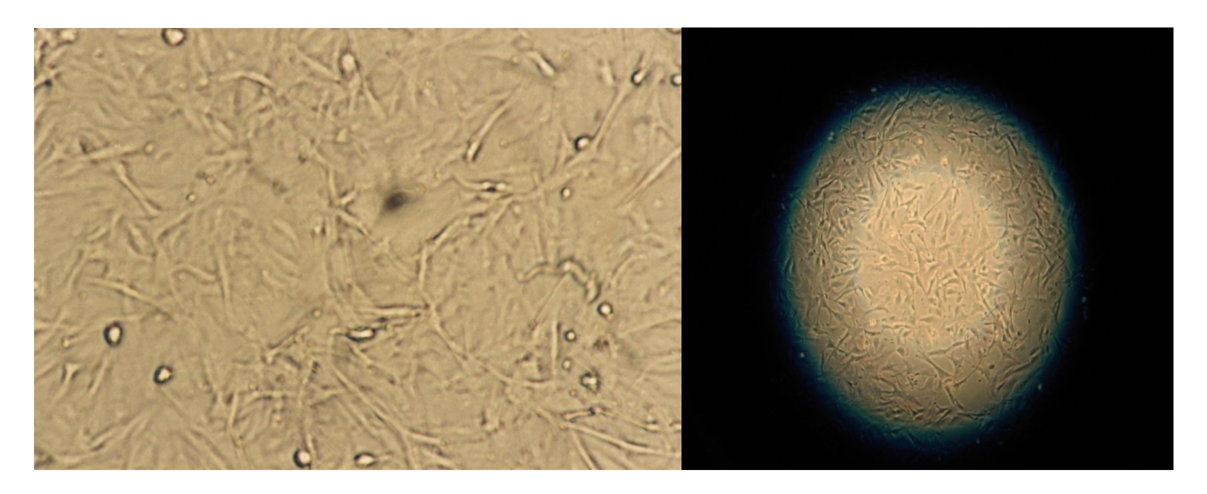


Fig. 8. Microscopic view (microscop Zeiss Axiovert 25C) of primary glioblastoma culture at passage (left) and 20 respectively (right).

During this project, we have purchased glioblastoma lines U-251 MG (initially named U-373 MG) from the European Collection of Cell Cultures (ECACC). This is one of the oldest and used cell-line of glioblastoma and it is very useful in this project because it assures a high occurrence of the events resulted from the experiments(4). This line was delivered in frozen criocilinders. For the revitalization we have used the usual revitalization process, and we have used the following compounds:

minimum essential medium(MEM), nonessential amino acid solution, piruvic solution, fetal serum, antibiotic solution, glutamine solution. U251 cell-line is passed at a confluence of 60-70%. The phenotypic aspect is shown is Fig.9

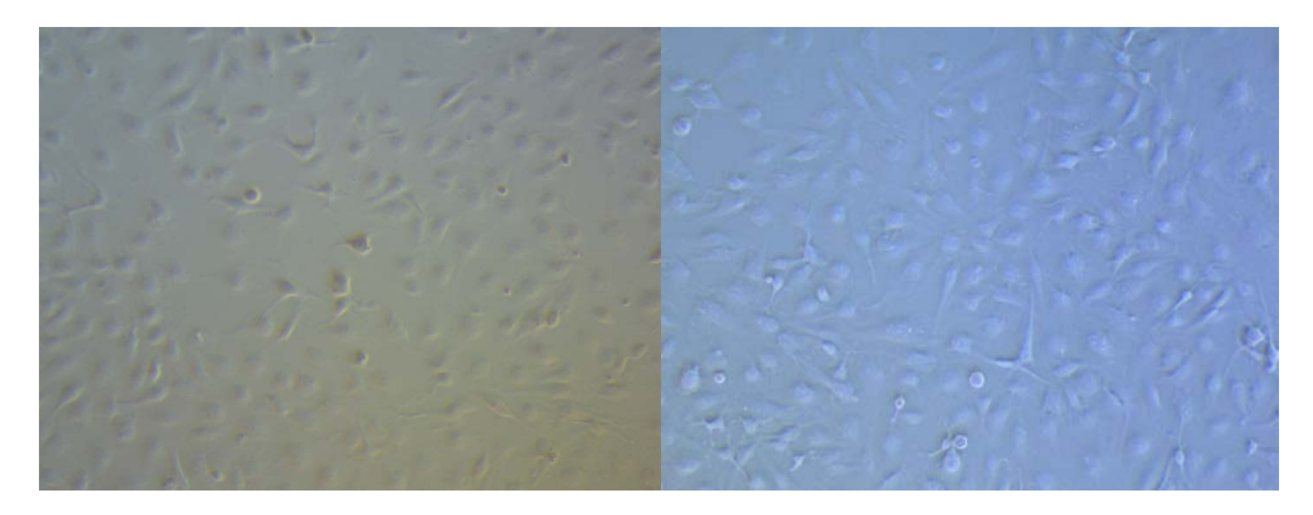


Fig.9. Microscopic aspect of U251 glioblastoma culture.

C. ARN extraction and biobank deposit

The samples were harvested from the patients, washed in PBS and stored in RNASave. The total ARN was isolated with the use of the Maxwell KIT 16 LEV simply RNA and Maxwell apparatus (Promega). In order to distinguish the RNA, the samples were mechanically fragmented and homogenized in tampon from the Maxwell 16 LEV simply RNA kit, with the help of a zirconium ball of 0,5mm diameter (circa 200mg). Homogenization was done in 2 cycles of 30sec each, separated by 1 30sec cooling cycle, using the Speed Mill (Analytik-Jena, Germany) apparatus. After centrifuge of the samples 1 min 5000g, 200uL of supernatant was mixed with 200uL of lysis buffer, strongly vortexed 15sec and inserted in the cartridge of the Maxwell 16 apparatus, where RNA isolation took place. The concentration of the RNA samples was evaluated by reading the optical density at 260nm at the Nanodrop spectrophotometer.

The isolated RNA quality was evaluated by determining the D0260/D0280 coefficient. All the samples had coefficients from 1.8-2, which indicates a high RNA purity isolated from the tissue. RNA with a weight ranging from 0.800 ug to 37 ug per probe was obtained. For reverse transcription of the qPCR we have used 500ng of RNA for each probe. RNA excess was deposited in a -80 degrees C biobank for further experiments.

D. qPCR analysis of genes involved in glioma invasion

In this project we have analyzed the expression of the following genes in the tumor tissue: PAFAH1B1(LIS1), NDEL1,CDK5, MYH9,TWIST1,SNAI2. LIS1, NDEL1 and CDK5 are components of the pro-neural pathway (molecular mechanism similar with the one utilized by the precursor neural bipolar migrating cells, during cerebral ontogenesis)(5,6), time in which TWIST1 and SNAI2 genes are part of the pro-mesenchymal components (molecular mechanism utilized by other types of tumor during metastasis)(7,8). MYH9 (myosin II) is a molecular engine proved to be involved in the migration of glial

tumor cells(5,6). As a reference expression we have used two housekeeping genes Actin B (ACTB) and GAPDH.

Reverse transcription RNA in cDNA were achieved using MMLV and oligo (invitrogen) and 500ng RNA, in a final volume of 50uL.

Real time PCR was achieved using the TaqMan Gene Expression Assays (Invitrogen) for the following genes:

- PAFAH1B1 (Assay ID: Hs00181182_m1),

- CDK5(Assay ID: Hs00358991_g1)

- MYH9 (Assay ID: Hs00159522_m1)

- TWIST1 (Assay ID: Hs01675818_s1

- SNAI2 (Assay ID: Hs00950344_m1)

- NDEL1 (Assay ID: Hs01092624_m1)

All probes for the aforementioned genes have been marked with FAM.

For the normalization of the results, we have analyzed the expression of the GAPDH and actin genes, and the probes belonging to them have been marked VIC.

The reaction mixture contained 1 uL of cDNA, 5uL TaqMan Universal Master Mix II, with UNG (concentrated x2m Invitrogen), 1uL primer and probes and 3ul water. Pipetting the probes on 384 wells clips has been achieved with the use of Qiagility(Qiagen) automated pipettor, using conductive 50uL tips.

The amplification programme was the following: 2 min,50 degrees C;10min,95 degrees C;followed by 40 cycles : 15 sec, 95 degrees and 1 min, 60 degrees was achieved in the 7900HT system apparatus from Applied Biosystem.

The results obtained in the SDS2.4 programme, were processed using the analysis RO Manager software.

The Values of the genes expression traced in the tumor and peritumoral(normal) samples are shown in table 1.

proba	nr. pac.	Assay	CDK5	MYH9	NDEL1	PAFAH1B1	SNAI2	TWIST1	ACTB vic	GAPDH vic
normal	1	1 (RQ)	0,9714	4,9686	1,867	2,251	1,2173	9,9229	1,4379	0,6955

normal	3	4 (RQ)	0,9471	1,9536	0,9278	1,5451	1,1274	0,1206	0,8542	1,1707
normal	4	6 (RQ)	1,2088	0,163	0,8194	1,5359	1,6445	0,351	1,2206	0,8193
normal	7	10 (RQ)	1	1	1	1	1	1	1	1
normal	8	12 (RQ)	0,6236	3,2145	3,475	3,0225	1,5478	0,3872	1,1074	0,903
normal	10	15 (RQ)	1,4261	2,0422	0,636	1,0868	2,5491	156,1858	0,9353	1,0692
normal	13	19 (RQ)	4,25	3,5051	2,1324	3,3684	0,7871	12,4159	0,9345	1,0701
normal	19	26 (RQ)	3,2193	3,1348	1,5566	1,8181	1,4803	1,7799	0,9797	1,0207
normal	20	28 (RQ)	0,822	1,983	1,0389	0,5797	1,3632	1061,1481	0,5873	1,7027
normal	21	30 (RQ)	0,8343	0,872	0,9905	1,2614	0,6833	1,0912	0,7298	1,3703
tumora	1	2 (RQ)	0,593	2,2895	0,3814	0,1078	2,8505	370,6255	0,4495	2,2246
tumora	3	5 (RQ)	1,3599	0,6167	0,3603	0,6792	1,3771	145,1248	0,6365	1,5711
tumora	4	7 (RQ)	2,2943	1,8173	0,9126	0,9301	3,2428	5,2325	1,1277	0,8868
tumora	7	11 (RQ)	1,9776	3,397	0,5937	0,5791	2,7548	0,067	0,5612	1,7819
tumora	8	13 (RQ)	1,6677	4,168	0,9616	0,8178	0,4639	24,3219	0,8966	1,1153
tumora	10	16 (RQ)	0,2629	3,7631	1,083	2,4531	6,457	246,7301	1,0814	0,9248
tumora	13	20 (RQ)	0,7655	1,022	0,9997	0,7724	1,3143	6,6413	1,1229	0,8905
tumora	19	27 (RQ)	1,2173	0,7826	0,4704	0,9973	3,3011	34,1066	0,4203	2,3793
tumora	20	29 (RQ)	0,9382	2,2798	1,0087	0,8262	1,5112	1107,3166	0,7897	1,2663
tumora	21	31 (RQ)	0,7491	3,8306	0,696	0,6982	2,5612	41,4521	0,8546	1,1701
tumora	9	14 (RQ)	0,8238	1,2496	1,0989	0,5959	3,0797	0,2886	0,573	1,7452
tumora	24	17 (RQ)	1,5311	0,7033	0,7352	0,566	3,5391	463,5058	0,509	1,9647
tumora	25	18 (RQ)	1,843	1,295	0,8638	0,8936	2,8642	14,3021	0,5292	1,8896
tumora	14	21 (RQ)	1,1479	1,3218	0,4402	0,7901		0,1735	0,5098	1,9617
tumora	15	22 (RQ)	0,6088	0,4047	0,4127	0,2448	2,6895	0,0735	0,4612	2,1683
tumora	16	23 (RQ)	1,7441	0,6171	0,7332	1,9885	3,5162	106,554	0,5555	1,8001
tumora	17	24 (RQ)	2,5625	0,7935	1,34	2,2342	0,7049	0,1133	0,4568	2,1894
tumora	18	25 (RQ)	1,6222	1,5868	0,8481	1,1109	0,8291	59,7247	0,6923	1,4444
tumor	2	3 (RQ)	1,871	1,9149	1,0235	0,6593	0,588	0,1668	0,6758	1,4797

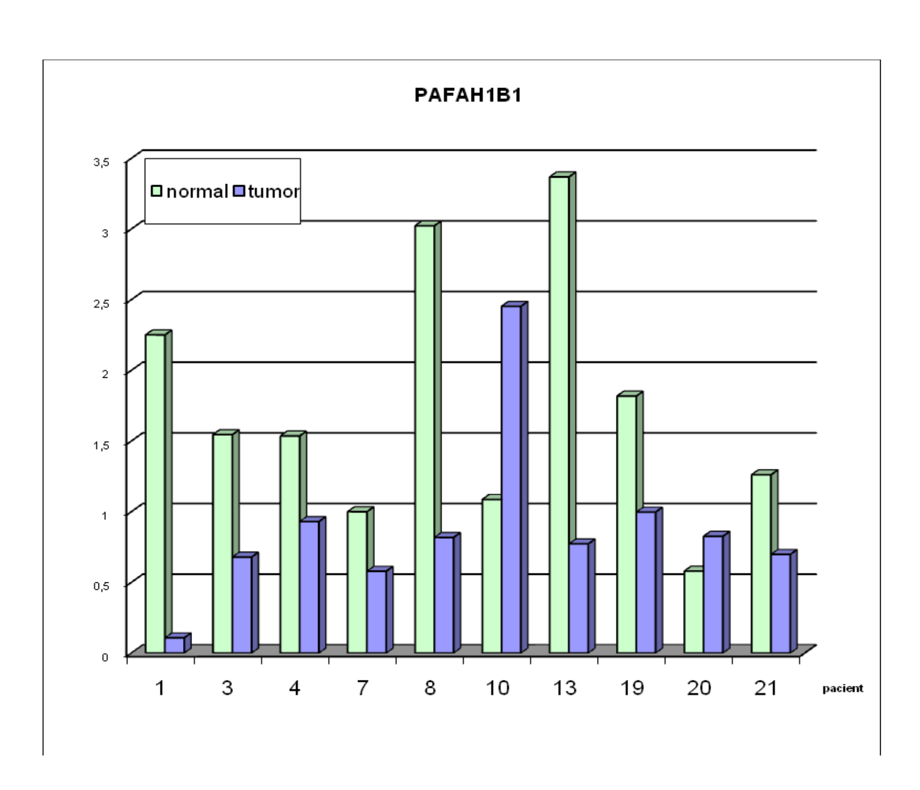
a			9				4			
tumora	5	8 (RQ)	4,0557	0,8265	4,1002	4,1463	7,2411	11,1016	3,1359	0,3189
tumora	6	9 (RQ)	0,1424	1,4381	0,5613	0,1722	1,2184	605,4901	0,5438	1,8388

Tabel 1. Gene expression in tumoral and normal samples

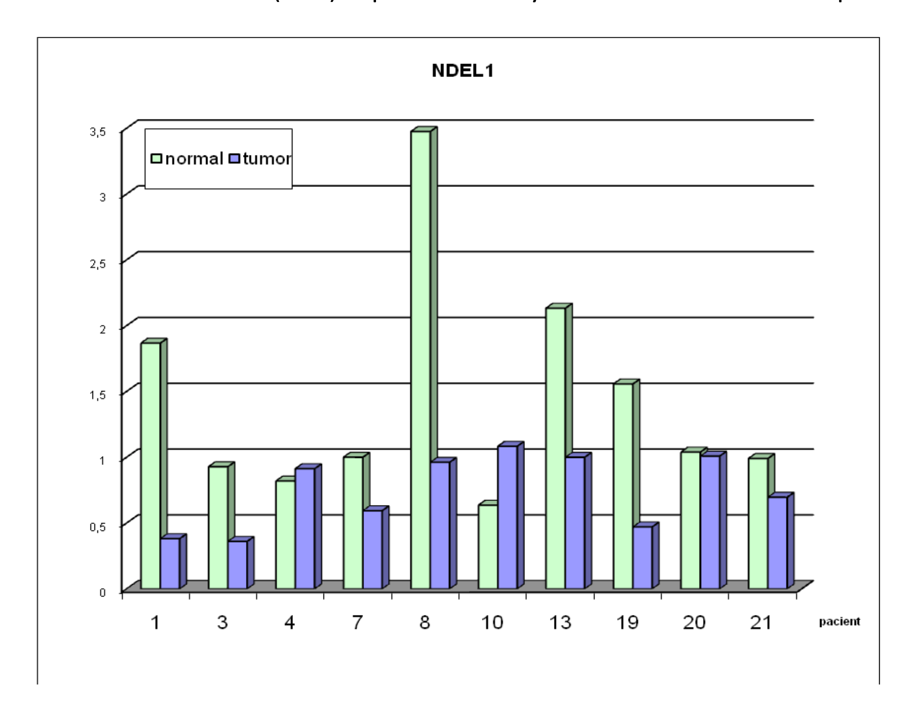
From the analysis from table 1 we can notice high values in certain samples tumoral and peritumoral (normal) for the TWIST gene, fact which makes it difficult to interpret the result obtained for this gene. Comparing the gene expression between tumoral and normal samples for the 10 patients from which we obtained both types is illustrated in table 2.

Nr. pacient	CDK5	MYH9	NDEL1	PAFAH1B1	SNAI2
1	**0,61**	**0,46**	**0,20**	**0,05**	**2,34**
3	**1,44**	**0,32**	**0,39**	**0,44**	**1,22**
4	**1,90**	**11,15**	**1,11**	**0,61**	**1,97**
7	**1,98**	**3,40**	**0,59**	**0,58**	**2,75**
8	**2,67**	**1,30**	**0,28**	**0,27**	**0,30**
10	**0,18**	**1,84**	**1,70**	**2,26**	**2,53**
13	**0,18**	**0,29**	**0,47**	**0,23**	**1,67**
19	**0,38**	**0,25**	**0,30**	**0,55**	**2,23**
20	**1,14**	**1,15**	**0,97**	**1,43**	**1,11**
21	**0,90**	**4,39**	**0,70**	**0,55**	**3,75**

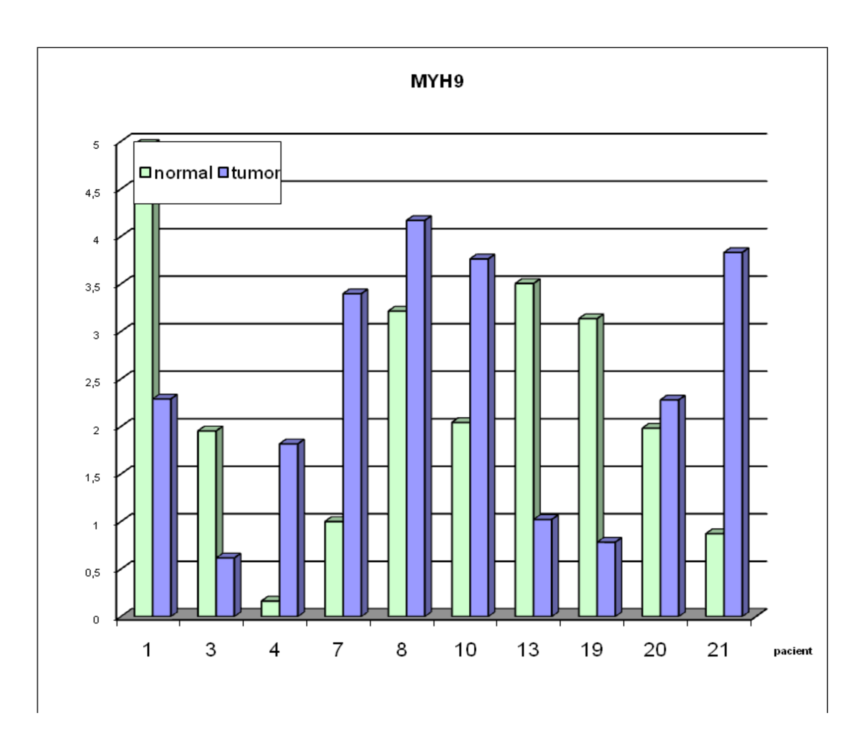
The graphic representation of the target genes in the tumoral tissue compared to the peritumoral for the 10 patients is shown in the following graphic.



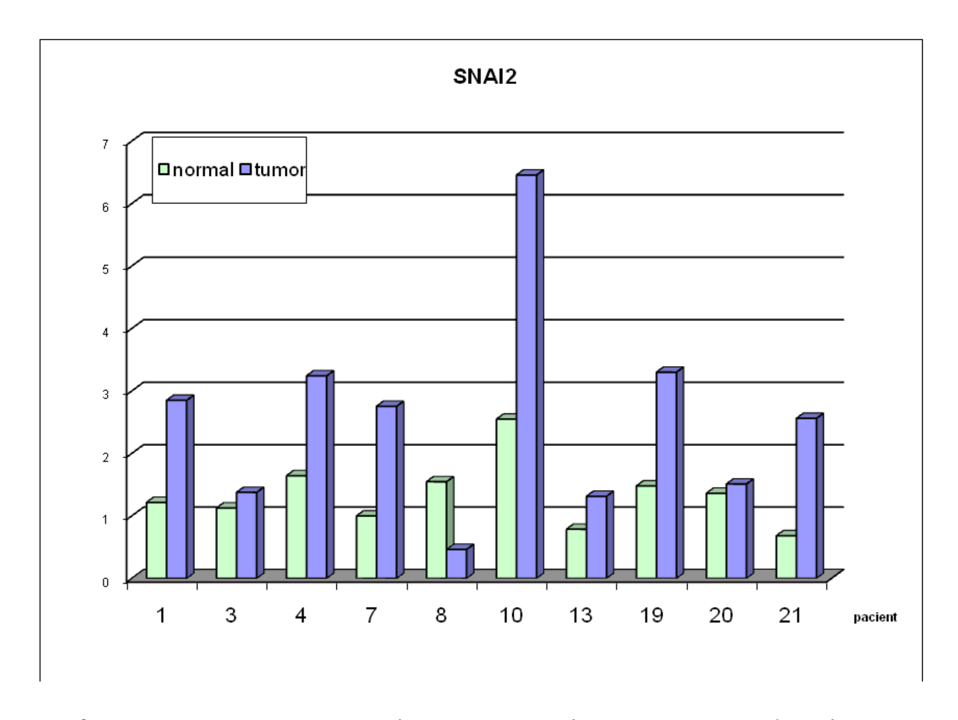
Grafic 1. PAFAH1B1 (LIS1) expression analysis in tumoral tissue compared to the peritumoral tissue



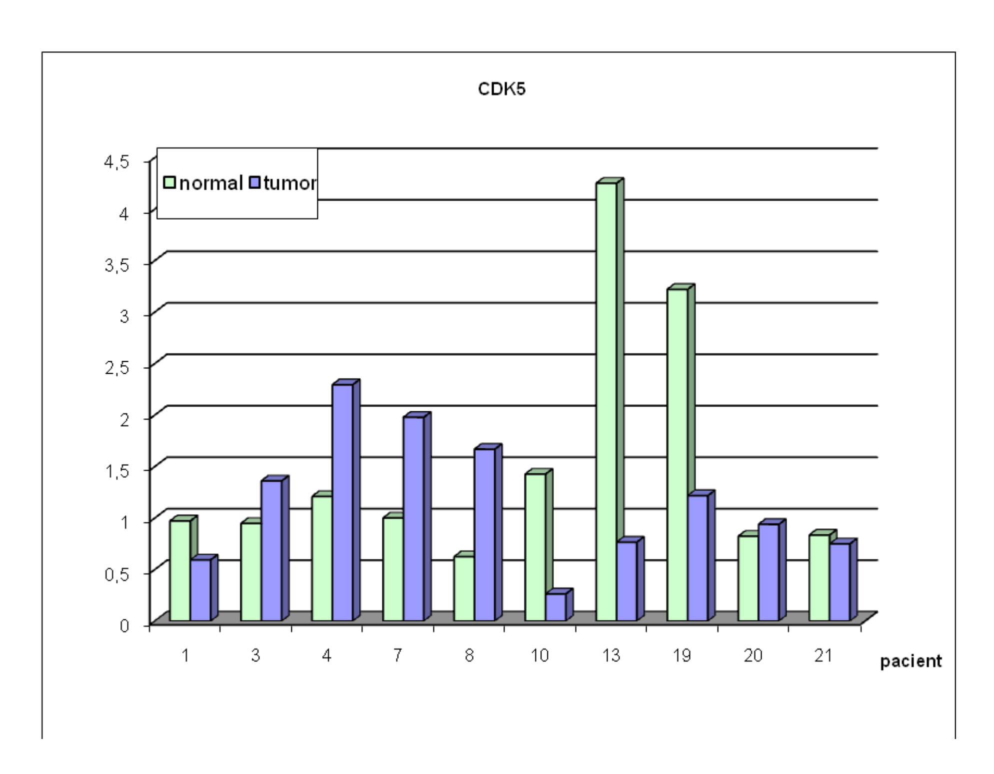
Grafic 1. NDEL expression analysis in tumoral tissue compared to the peritumoral tissue



Grafic 3. MYH9 expression analysis in tumoral tissue compared to the peritumoral tissue



Grafic 4. SNAI2 expression analysis in tumoral tissue compared to the peritumoral tissue



Grafic 5. CDK5 expression analysis in tumoral tissue compared to the peritumoral tissue

From the analysis of the target genes in the 10 cases at which we could harvest tumor samples as well as peritumoral (normal) samples, we have state that the SNAI2 gene that presents a constantly high activity in all tumoral samples except in case nr,8 (graphic 4). MYH9 gene present a high activity in 6 out of the 10 cases, CDK5 genes in 5 out of 10 cases and LIS and NDEL1 in 2 out of 10 cases.

Comparing the average expression of the target genes (except TWIST gene) in the tumoral tissue (21 samples) and peritumoral cerebral (10 samples) we have obtained the following results (table 3). We can observe a higher expression of the SNAI2 in tumor samples (with 95%) comparing the normal samples. SNAI2 gene is involved in the oncogenetic mechanism, specially in the invasion and metastasis of the carcinomas and we also proved the its high expression in malignant gliomas (10) as well as the correlation of the TWIST expression.SNAI2 gene may present as a molecular target in the anti invasive therapy and will be studied in the following steps of the project. For the TWIST gene we shall repeat the qPCR analysis.

Tabel 3: Average gene expression of genes in normal and tumoral samples

Medie	CDK5	MYH9	NDEL1	PAFAH1B1 (LIS1)	SNAI2
N	1,52	2,28	1,44	1,74	1,33
T	1,42	1,71	0,93	1,05	**2,60**

The expression of the genes involved in the pro-neural path is elevated in the cerebral tissue compared to the tumoral tissue. For the CDK5 gene this result was emphasized by other authors which noticed the CKD5 level in glioblastoma is lower (at a small difference, statistically insignificant) than the cerebral tissue, but is larger compared to the astrocit cells. This is due to fact that CDK5 levels is usually higher in neurons compared to astrocytes, thus knowing the fact that astrocit cells are the origin for grade II-IV gliomas, we can conclude that CDK5 is overstated in glial tumor tissue. This mechanism could explain other results achieved for the other genes involved in the pro-neural pathway (LIS1 and NDEL1) in which we have elevated expression in the cerebral tissue compared to the tumoral tissue.

E. Processing the extracted tissue samples, cultivated after the “organotypic brain slices” model and evaluation of the viability after the culture

One of the experimental models frequently used for the study of invasive gliomas is the in tissue model (Organotypic Brain Slice Culture) of cultivation for cerebral mouse tissue sections. This model does not reflect sufficiently enough the in situ biological reality. Thus there are differences between cellular morphology and characteristics of the mouse compared to the human and, moreover, at the periphery of the tumor occur some phenomenons such as : peritumoral edema, peritumoral gliosis, which influence the migration of tumoral cells. So a new element of the project is the development of a new in tissue model in glioblastoma invasion. For this purpose we have harvested using stereotactic tisular fragment that include the tumoral as well as the peritumoral portion which represents the transition area between normal cerebral tissue and tumor which is very important from a glioma invasion point of view. The initial target was to localize at the periphery of the hypersignal T1 contrast MRI area, for grade III and IV gliomas, as well as the periphery area of the hypersignal in the FLAIR sequence for grade II gliomas. Therefore the needle used for the biopsy was Sedan with a 10mm hole, on the same tissue sample we could identify at the histopathological analysis the tumoral tissue as well as the peritumoral cerebral tissue (Fig.9).

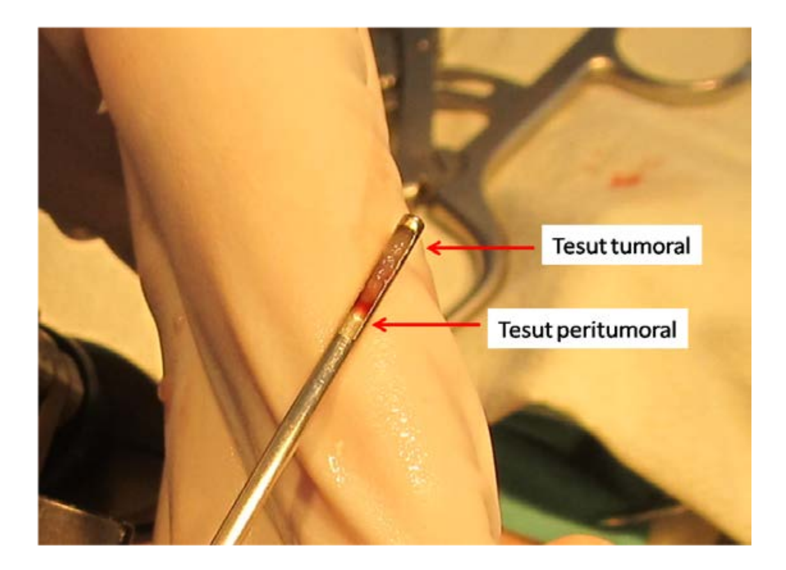


Fig.9. Intraoperative view of tissues samples obtained by stereotactic biopsy.

Tissular Cylinder was sectioned using the Mcllwain Tissue Chopper microtome in tissular section of approx 300-400 microns in depth. The sections were cultivated on specially treated culture clips (flat culture clips with 0.4 microns pores and a 12 and 30mm diameter), using the DMEM environment supplemented with glutamine fetal serum and AB solution at a temperature of 37 degrees C and CO2 5%. On a period of 14 days we have observed the viability of tissular sections, with the preservation of the cellular architecture. The study of tumor-cell migration using tissue sections extracted from the tumor periphery and cultivated in vitro will be achieved in the following step of the project.

II. During the second part of the project, the following objectives and activities have been accomplished in the completion of the project :

Objective 1. Evaluation of the efficiency of a new experimental invasion model in gliobmastoma: "organotypic brain slices" type model.

Activity 1.1 Inoculation of lines and primary cultures of glioblastoma at a tissue level and monitorization of tumor cells through fluorescent microscopy

Activity. 1.2 Evaluation of a new experimental invasion model compared to the existing ones.

Objective 2. Blocking of the gene/molecule involved in glioma invasion – evaluation through "scrape migration assay" technique.

Activity 2.1 Targeted gene/molecule blocking in glioblastoma lines and primary cultures.

Activity 2.2. Evaluation of the inhibitory efficiency of migration through " scrape migration assay" (surface migration, two dimensions)

Moreover, apart from the activities planned for the second phase of the project, we have continued the activities from the first objective of the first project, such as evaluation of the genes involved in the invasion of the glioblastoma samples through qPCR, in the purpose of raising the glioblastoma samples, taken into work and obtaining statistical meaningful results.

We shall describe the initial results through continuing the activities from the 2nd objective of the first phase, and then we shall describe the activities for 2014 in detail.

A. Evaluation of the genes expression involved in glioma invasion in glioblastoma samples through qPCR analysis.

The protocol for sample harvesting, of mRNA extraction and the qPCR analysis have been described in detail in the reporting at the end of phase I /2013. Holding account of the data already existed in specialty literature, their expression has been analyzed in the tumor tissue, the following genes: PAFAH1B1 (LIS1), NDEL1,CDK5,MYH9,TWIST1,SNAI2(5-8,13-16). Because it has been proven that an

isoform of GFAP characteristic from the sub ventricular cerebral area (17), mentioning that this rationing has been explored in this project, the expression of some genes specific to precursor neuro-migration, such as LIS1 and myosin II(5,6,14). As a reference expression, 2 house-keeping genes Actine B(ACTB) and GAPDH. During phase I we managed to analyze through qPCR the expression of the mentioned genes for the 31 samples, out of which 10 were normal and 21 tumoral. In the year 2014, there have been introduced new tumor probes, therefore the total number of samples has reached 69, out of which 14 are normal and 55 tumor. The result of the qPCR of the genes taken into study are presented in the table below (Table 1).

normalization			actin	actin	GAPDH	actin	actin	actin
Sample	Sample	Sample	SNAI2	LIS	TWIST1	CDK5	MYH9	NDEL1
N	n1	1	0,6749327	1,1	0,1078667	1,1494912	2,8655238	1,7760257
N	n4	4	0,9462516	1,3788933	0,3066562	1,1740528	1,3451107	0,8012999
N	n6	6	1,153662	1,9943894	0,2706523	1,5002879	0,8551787	0,7951428
N	n10	10	0,78418565	2,4631116	0,3625726	0,9892368	1,2383419	1,1335291
N	n12	12	0,97599185	2,3525767	0,3028768	1,1885175	2,0488303	1,9741436
N	n15	15	2,1888669	0,69336116	57,029984	1,0856664	0,9782558	0,5261313
N	n19	19	0,51876605	2,4652798	0,3525888	1,356065	3,440769	2,257662
N	n26	26	1,095774	1,4440179	2,985024	1,3963466	1,2793784	1,1332624
N	n28	28	2,5241377	1,2284328	227,57576	0,9656269	2,012856	1,3490385
N	n30	30	1	1	1	1	1	1
N	n35	35	0,5348164	1,516513	0,2745844	1,2567084	3,439325	1,9086698
N	n50	50	0,31063914	0,6378862	0,2237834	1,2324991	1,7388849	0,3991498
N	n60	60	0,33678085	0,7424016	2,4950075	1,0844022	2,2522898	0,8750492
N	n69	69	1,5816196	0,39283273	11,603235	1,3208619	1,6366099	0,358656
G4	g4-2	2	3,192736	0,89717144	19,57778	0,2258378	1,2911986	0,4536128
G4	g4-5	5	1,9088831		10,790553	0,8998336	0,5791326	0,3439418
G4	g4-7	7	3,5717516	1,8053235	1,1385132	1,7938024	1,111368	0,6623674
G4	g4-9	9	3,3443635	0,8000256	1,7169864	0,0461709	3,3348596	1,6163797
G4	g4-13	13	0,4703629		4,878259	1,7439592	2,351938	0,7867881
G4	g4-14	14	3,422031	0,6709355	1,4012685	0,649862	1,0415183	0,4582166
G4	g4-16	16	2,8646147	0,9722431	10,681849	0,5488121	2,3003132	0,597667
G4	g4-17	17	4,6518636		77,985466	0,7772149	0,5926827	0,8300147
G4	g4-18	18	2,8478541	0,8877553	4,585317	0,8892837	0,6764384	1,0514443
G4	g4-20	20	1,4991231	0,78355414	3,1646574	0,7524809	2,0253797	1,0141844
G4	g4-21	21	29,245	1,7030469	0,2385029	0,6575629	1,5128391	0,9267684
G4	g4-22	22	3,988003	0,5921764	0,7719408	0,4488208	0,4927271	0,4272901

G4	g4-25	25	0,5564403	1,8502196	11,955679	1,0858573	0,5741252	0,6830797
G4	g4-27	27	4,4155273	0,86378944	61,336624	0,6625951	0,7126675	0,7138382
G4	g4-29	29	1,6148674	0,7816416	79,65067	0,4568896	1,3301022	0,9025892
G4	g4-36	36	4,62623	0,45391524	40,242218	0,8403888	2,3201578	0,4031062
G4	g4-40	40	3,0652282	0,7734155	4,408994	1,369331	2,4048038	0,336806
G4	g4-41	41	1,4157643	0,33492115	0,143188	0,4639296	1,5577198	0,3553785
G4	g4-43	43	0,29047558	0,9438865	5,220161	1,7207627	3,4705014	2,0440834
G4	g4-46	46	4,637617	0,43701303	13,427031	2,5740855	4,122891	0,4235704
G4	g4-47	47	3,483697	0,38275817	20,295422	0,6928809	3,271392	0,4530459
G4	g4-48	48	3,47046	0,47063917	1232,1271	1,3545989	3,8288715	0,3483994
G4	g4-49	49	4,5127873	0,4160622	86,97465	1,184777	3,32765	0,267408
G4	g4-51	51	0,16274476	0,2458869	2,66E-08	0,6900988	2,7792144	0,0587766
G4	g4-55	55	4,67951	0,4962683	36,11937	1,5336149	0,757222	0,4876883
G4	g4-57	57	1,7145797	0,51221544	0,2282752	0,8638431	1,6642057	0,2606624
G4	g4-58	58	16,60257	0,8801585	613,81116	1,1665877	3,7830327	0,7662422
G4	g4-62	62	14,095978	0,32623896	613,81116	1,1665877	3,7830327	0,7662422
G4	g4-66	66	8,9248495	0,43509337	1215,6658	1,749158	5,3047953	0,6837325
G4	g4-67	67	2,4027183	0,8211608	81,282104	1,6472945	0,8262585	0,6634226
G4	g4-68	68	1,2693882	0,21300572	29,341976	0,6245123	4,198156	0,1870508
Gg3	g4-42	42	64,569145	0,51125443	3349,8125	1,3105843	0,6540021	0,8123602
G4	g4-63	63	5,634425	0,26670825	17,853788	0,1806208	3,59724	1,2749629
G4	g4-56	56	2,5002875	0,3134505	172,46667	1,7414806	1,1930535	0,1850922
A4	a4-23	23	4,1156287	1,8147094	6,0058503	1,1670161	0,4482096	0,7928963
A3	a4-11	11	2,900983	0,6802351	0,0050859	0,9061351	2,470877	0,4491975
A3	a4-39	39	2,9706857	1,0391644	92,1751	0,761427	3,2198162	1,1631088
A2	a4-3	3	0,44225997	2,6043985	0,2423265	2,0375364	1,0620816	1,1646429
A2	a4-8	8	1,2081627	4,2439437	2,9952412	2,3658884	0,7651555	0,9273132
A2	a4-32	32	0,20543556	1,639407	5,212275	1,9971213	1,1563962	0,8535538
A2	a4-34	34	0,5191022	1,6104385	0,1750345	1,1364878	1,2806332	0,7613568
A2	a4-65	65	0,18945585	0,9029921	3,2385302	2,1115248	1,9727054	1,2287071
A	a4-59	59	0,66915	0,8856938	25,712393	4,702592	2,0853221	0,7689731
HTB14	HTB14	HTB14	23,63285	0,19919638	191,79683	0,7297989	5,111779	2,015784
G4	GM-54	54	7,1417303	0,21249886	0,0641615	1,839141	1,902634	0,1759097
Ge	GE-31	31	1,3391132	0,33470514	77,37963	0,6478979	1,4708928	0,4290154
AP1	AP1-61	61	1,0472172	0,7123309	3,4022565	1,271499	3,6442165	0,7738772
OG3	OG3-44	44	7,9978375	1,9566491	1,0516542	5,275719	1,2407566	1,4662437
OG3	OG3-52	52	0,89612865	0,83994657	0,2013261	1,1578295	1,9832059	0,8628495
OG2	OG2-64	64	2,6950877	0,32492846	208,10793	6,3470855	1,1222928	0,5144352
Og	OG-24	24	0,52404284	2,1186123	0,083276	0,6813225	0,8916391	1,412557
Oa2	OA2-33	33	0,23408303	0,659317	4,0587144	2,121049	0,8875228	0,3219472
Oa2	OA2-37	37	0,3146258	1,3507082	18,182789	1,5715398	2,4422646	1,5082309

G4	???-70	70	5,2001953	0,220992	171,6732	1,182272	4,8172584	0,4143169
TPE	TPE-45	45	1,3523018	1,4100994		2,229959	2,526271	

Table 1. Expression of the SNAI2, LIS1, TWIST1, CDK5, MYH9(myosin II, NDEL1 quantified throught qPCR in the tumoral tissue (55 samples) compared to normal tissue (14 samples). Legend : N-normal,G4-gliobalstoma grade IV, A3- anaplastic astrocytoma grade III, A2 – diffuse astrocytoma grade II, AP – pilocytic astrocytoma grade I, OG3 – anaplastic oligodendroglioma grade III, OG2- grade II oligodendroglioma, Oa2 – grade II oligoastrocytoma , TPE – primitive ectodermal tumor, Ge-germinoma,GG3- grade III anaplastic ganglioglioma.

In the following graphics there have been expressed the following genes taken into the study : SNAI2 (Fig,.1) ; LIS1 (Fig.2); TWIST 1(Fig.3); CDK5(Fig.4);MYH9/myosin II (Fig.5) and NDEL1(Fig.6).

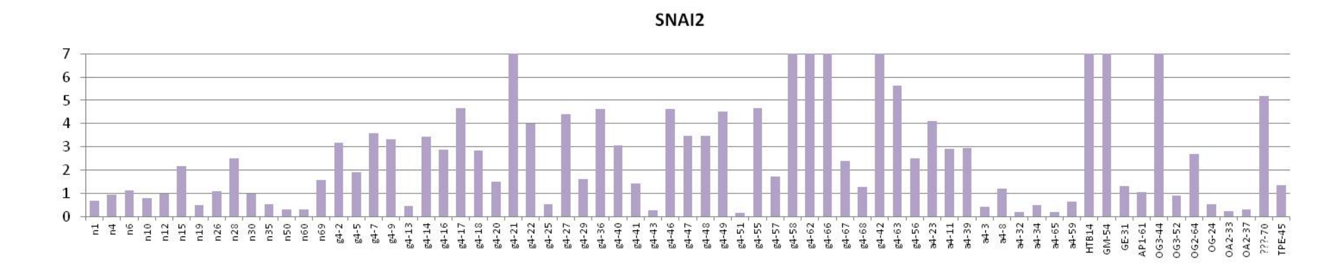


Fig.1. The graphic of the values expressed by the SNAI2 gene, evaluated through qPCR of the normal and tumor tissue

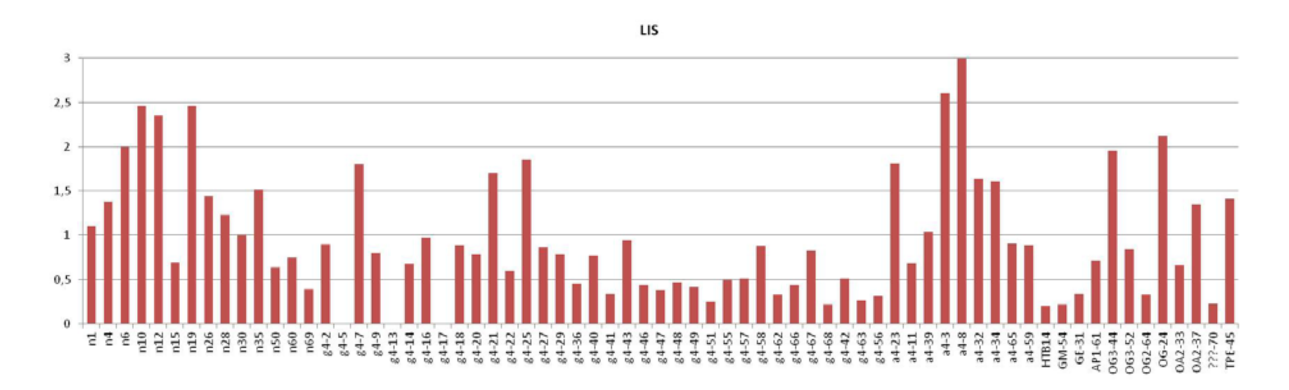


Fig.2. The graphic of the values expressed by the LIS 1 gene, evaluated through qPCR of the normal and tumor tissue

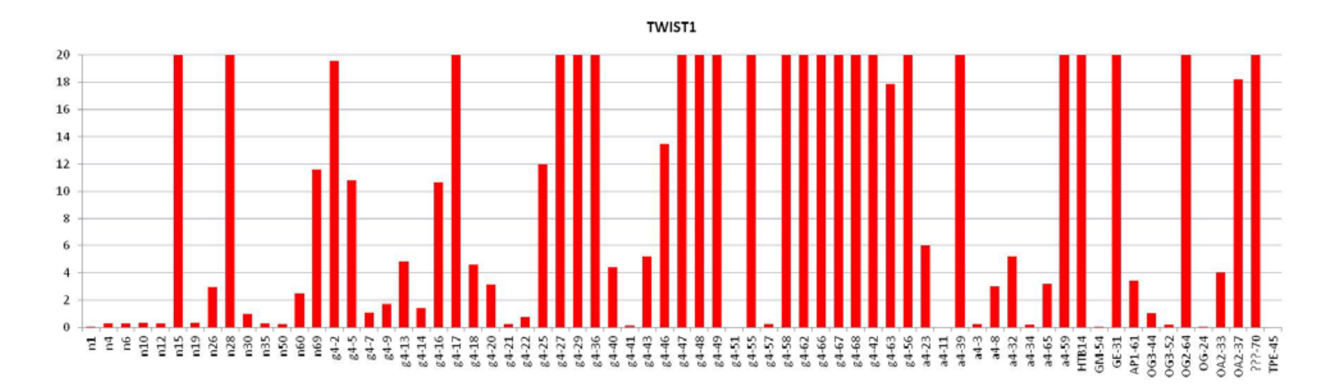


Fig.3. The graphic of the values expressed by the TWIST1 gene, evaluated through qPCR of the normal and tumor tissue

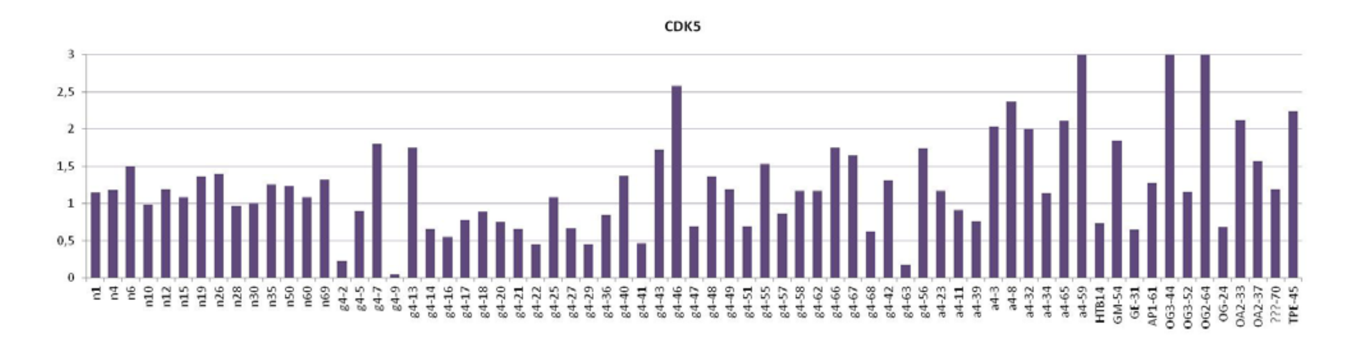


Fig.4. The graphic of the values expressed by the CDK5 gene, evaluated through qPCR of the normal and tumor tissue

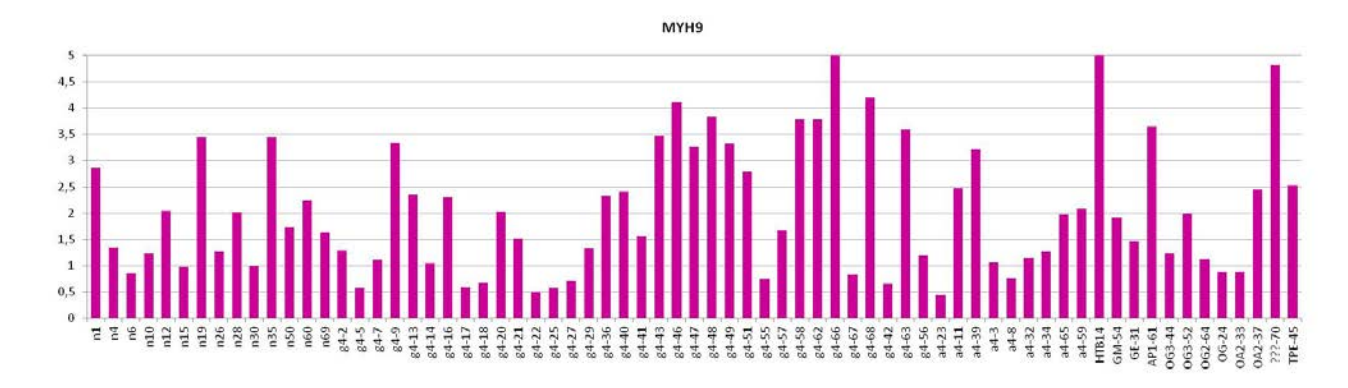


Fig.5. The graphic of the values expressed by the MYH9/myosin II gene, evaluated through qPCR of the normal and tumor tissue

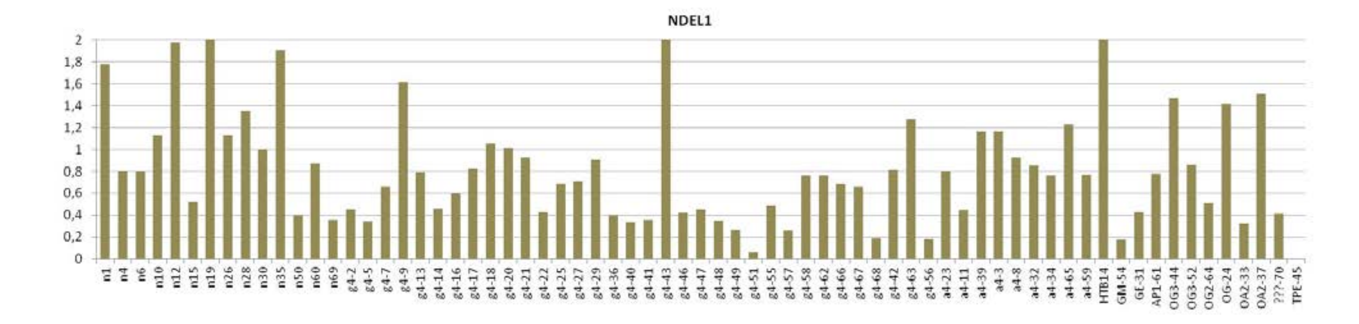


Fig.6. The graphic of the values expressed by the NDEL1 gene, evaluated through qPCR of the normal and tumor tissue

Calculating the average of the expression of the genes taken in to study between normal samples and glioblastoma (grade IV) and astrocitoma (grade II), we notice a big difference for the SNAI2 and TWIST genes, thus confirming for the 2nd time the preliminary data obtained in phase 1. (Table 2)

mean	normal	Glioblastoma (grad IV)	Astrocitoma (grad II)	**st dev**	n	g	a
SNAI2	1,044745	6,342702	1,468985	**SNAI2**	0,656225	11,68792	1,466646
LIS	1,386407	0,704579	1,713443	**LIS**	0,698653	0,427125	1,122929
TWIST1	21,7779	230,0913	15,08465	**TWIST1**	61,11445	633,3267	29,98384
CDK5	1,19284	1,015121	1,909525	**CDK5**	0,161813	0,566112	1,201467
MYH9	1,866525	2,140338	1,6068	**MYH9**	0,868088	1,35468	0,892187
NDEL1	1,163411	0,6543	0,901083	**NDEL1**	0,609924	0,41066	0,250322
TWIST1	1,037043	10,95673	0,718317	**TWIST1**	2,910212	30,15841	1,427802

Tabel 2. The average of genes expression between normal tissue and grade IV glioblastoma and grade II astrocytoma

Noticing this difference, we have made a separate statistical analysis for the two genes (TWIST1 and SNAI2), comparing only their expression between the normal and tumor samples. We have excluded other non-glioma tumors(geminoma,primitive neuroectodermic, etc.). The table of the two genes expression is exposed below. Moreover, we have analyzed the expression of qPCR of the two genes in the glioblastoma cell-line HTB14(U87) that we are using in the project.

Sample	Number	SNAI2	TWIST1
N	1	0,6749327	0,1078667
N	4	0,9462516	0,3066562
N	6	1,153662	0,2706523

N	10	0,7841857	0,3625726
N	12	0,9759919	0,3028768
N	15	2,1888669	57,029984
N	19	0,5187661	0,3525888
N	26	1,095774	2,985024
N	28	2,5241377	227,57576
N	30	1	1
N	35	0,5348164	0,2745844
N	50	0,3106391	0,2237834
N	60	0,3367809	2,4950075
N	69	1,5816196	11,603235
G4	2	3,192736	19,57778
G4	5	1,9088831	10,790553
G4	7	3,5717516	1,1385132
G4	9	3,3443635	1,7169864
G4	13	0,4703629	4,878259
G4	14	3,422031	1,4012685
G4	16	2,8646147	10,681849
G4	17	4,6518636	77,985466
G4	18	2,8478541	4,585317
G4	20	1,4991231	3,1646574
G4	21	29,245	0,2385029
G4	22	3,988003	0,7719408
G4	25	0,5564403	11,955679
G4	27	4,4155273	61,336624
G4	29	1,6148674	79,65067
G4	36	4,62623	40,242218
G4	40	3,0652282	4,408994
G4	41	1,4157643	0,143188
G4	43	0,2904756	5,220161
G4	46	4,637617	13,427031
G4	47	3,483697	20,295422
G4	48	3,47046	1232,1271
G4	49	4,5127873	86,97465
G4	51	0,1627448	2,66E-08
G4	55	4,67951	36,11937
G4	57	1,7145797	0,2282752
G4	58	16,60257	613,81116
G4	62	14,095978	613,81116
G4	66	8,9248495	1215,6658
G4	67	2,4027183	81,282104

G4	68	1,2693882	29,341976
G4	63	5,634425	17,853788
G4	56	2,5002875	172,46667
G4	23	4,1156287	6,0058503
G4	70	5,2001953	171,6732
G4	54	7,1417303	0,0641615
G3 (og)	59	0,66915	25,712393
G3	42	64,569145	3349,8125
G3	11	2,900983	0,0050859
G3	39	2,9706857	92,1751
G3 (og)	24	0,5240428	0,083276
G3 (og)	52	0,8961287	0,2013261
G3 (og)	44	7,9978375	1,0516542
G2	3	0,44226	0,2423265
G2	8	1,2081627	2,9952412
G2	32	0,2054356	5,212275
G2	34	0,5191022	0,1750345
G2	65	0,1894559	3,2385302
G2 (og)	64	2,6950877	208,10793
G2 (oa)	33	0,234083	4,0587144
G2 (oa)	37	0,3146258	18,182789
G1	61	1,0472172	3,4022565
HTB14	60	23,63285	191,79683

Tabel3. Expression of the SNAI2 and TWIST1 quantified through qPCR in glioma samples – grade IV gliobastoma (36 samples) compared to grade III glioma (7 samples), grade II (8 samples , grade I (1 sample), glioblastoma cell line HTB14(U87). Legend : N-normal,G4-gliobalstoma grade IV, A3- anaplastic astrocytoma grade III, A2 – diffuse astrocytoma grade II, AP – pilocytic astrocytoma grade I, OG3 – anaplastic oligodendroglioma grade III, OG2- grade II oligodendroglioma, Oa2 – grade II oligoastrocytoma , TPE – primitive ectodermal tumor, Ge-germinoma,GG3-grade III anaplastic ganglioglioma.

We have done the statistical analysis of the expression of the SNAI2 and TWIST1 genes, through qPCR, comparing this results between samples, normal tissue (14 samples), grade IV glioma (36 samples), grade III glioma (7 samples), grade II glioma (8 samples), grade I glioma (1 sample). The sample that came from the gliombastoma cell-line has not been included in the statistical calculus.

- The difference between tumor tissue (including all glioma samples included in ths tudy – 52 samples) and normal tissue (66 samples). We have used the Kolmogorov-Smirnow test to verify the distribution of TWIST1 and SNAI2 values. Distribution of the SNAI2(mean-4,08 standard deviation=8,76) ($p<0.001$) and TWIST (mean=131.37, standard devioation=464.67) are not normal ($p<0.001$). Keeping in mind this result, we have used non-parametrical tests (Mann-

Whitney test). Using this test, we notice that mean rank=37.00 of SNAI2 expression is statistically bigger that in normal tissue (mean rank=20,5) (U=182,00, p=0,004). Also, mean rank=36,17 of the TWIST1 in tumor samples was bigger than the normal tissue (mean rank=23,57) (U=225,00 , p=0.029).

- The difference between tumor grades (52 samples). There haave been used non-parametrical Kruskal-Wallis H, which showed the existence of semnificative statistical values of SNAI2 between samples of different grades (chi square =14,42 , p<0.001). Therefore, mean rank of SNAI2 score was 14,00 for grade I, 8,75 for grade II, 27.00 for grade III, and 30,69 for grade IV. Although there are big differences between mean rank a TWIST1 of the grade I-IV samples, they are not statistically meaningful (chi square=2.313, p=0.510), with a mean rank of 19.00 for grade I, 21.63 for grade II, 22,29 grade III, and 28.61 grade IV.

The difference between the mean values of the SNAI2 and TWIST 1 expression in the normal tissue and the tumoar tissue are illustrated in Fig.7 and Fig.8.

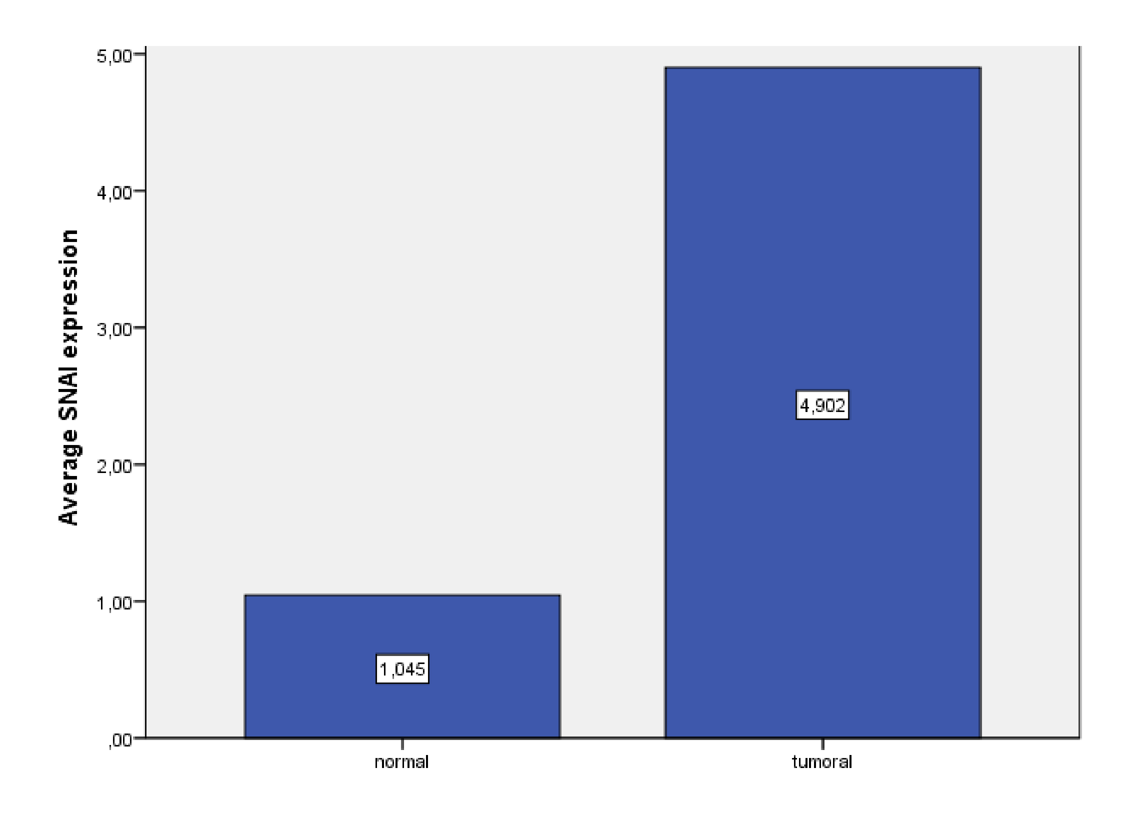


Fig. 7. Average SNAI2 expression using qPCR in normal and tumor samples

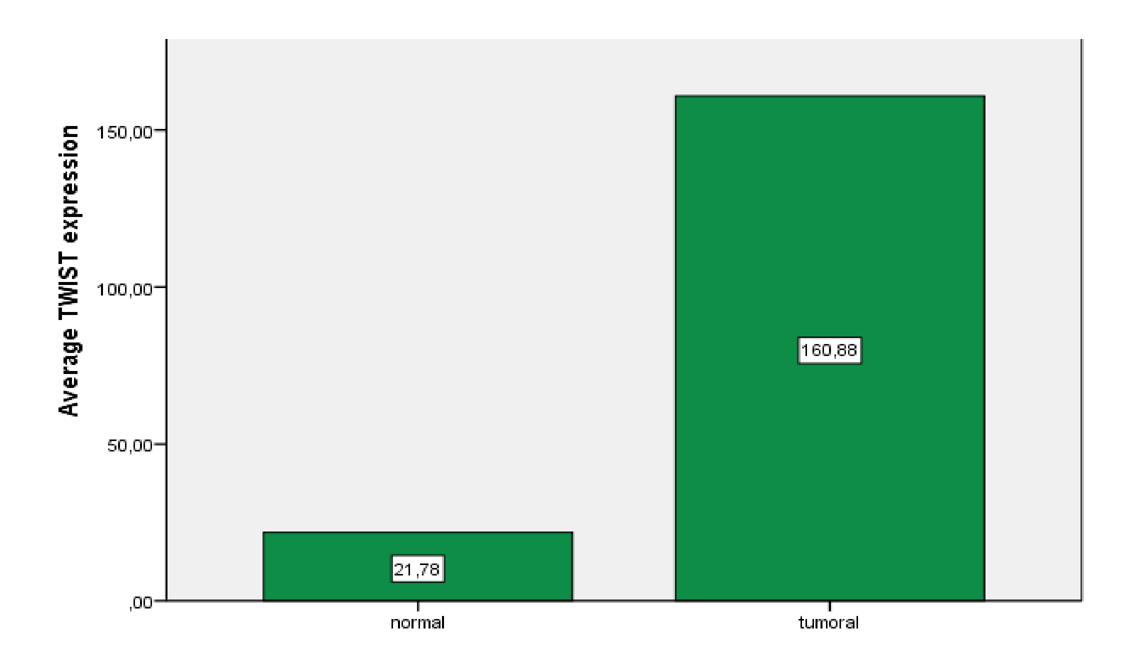


Fig. 8. Average TWIST1 expression using qPCR in normal and tumor samples

The result obtained from this phase confirm the preliminary result from the previous phase (phase I). Having a larger number of samples compared to the previous phase, we could obtain data of high statistical value, which showed that two of the analyzed genes, SNAI2 and TWIST1 are over-expressed in cerebral gliomas. Moreover, for the SNAI2 genes we could notice a direct link between the malignancy grade and the gene expression, the expression being the highest in grade IV gliomas, the most malignant glioma. These results agree with the ones that were recently published in the specialized literature TWIST,SNAI. Holding to account that there already is data that suggests the involvement of these genes in glioma invasion (5,6,13), they may become potential targets for the anti-invasive molecular therapy in glioblastoma. For the LIS1 gene, which is going to be studied, according to the project proposal of financial acceptance, it has not been observed an over-expression in tumor samples compared to normal. This result is not like the observations published in the only article the authors could find that evaluate the LIS1 expression in cerebral gliomas(14). Thus, the authors of the article noticed at a level of proteical expression (Western-blot analysis) that the expression of the LIS1 in cerebral tissue is similar to the on in tumor tissue. Imunohistochemical analysis suggests otherwise. If the normal cerebral tissue express a diffuse pattern of the LIS1 protein, at the tumor sample analysis(malignant, grade III-IV gliomas), there is a high expression of LIS1 at the infiltrative tumoral cells, whereas the normal uninfiltrative tumor tissue do not express this protein. Keeping in mind these observation, during objective II of this phase, we have achieved the inhibition of LIS1 gene through shRNA transfection, according to the objectives presented in the project proposal that was accepted for financing, followed by a detailed study of the SNAI2 gene role, gene which proved to be over-expressed in tumor samples compared to normal samples.

B. Activities developed during the second part of the project

Objective 1. Evaluation of a new experimental model of invasion in glioblastoma: „organotypic brain slices” model

Activity 1.1. Inoculation of glioblastoma lines at brain tissue sections and monitoring the migration of tumoral cells by fluorescence microscopy.

Activity 1.2. Evaluation of efficiency of new model comparing with the present ones

In order to evaluate the migration of tumoral cells, it is necessary to develope an experimental model which enable the researcher to visualize the tumoral cells in tissue. During this part of the project we develop such a model and established the following activities: obtaining the cerebral tissue sections, inoculation of glioblastoma cells, and monitoring the migration of GFP tumoral cells by fluorescent microscopy. We used the NMRI mice of 3-6 day-old. The experiments were conducted according with the national and European legislation and with the approval of the Ethic Committee of "Bagdasar-Arseni" Clinical Hospital.



Fig.9. Phenotypic aspect of the NMRI mouse.

For mice anesthesia we used 1 mg de fenobarbital injected intraperitoneally. This doze induce to the mouse a deep coma and analgesia. After coma induction, te mouse is immersed in ice water for 3 minutes and after that the surgical techniques of cerebral extraction are initiated. We mention that this technique is similar with the existing protocols (5,8,12). However there are several personal nuances. Therefore we chose to induce the barbituric coma, followed by deep hypothermia. These combined techniques deeply reduce the metabolism of cerebral tissue, maintaining the viability of the tissue during the surgical procedure. The surgical procedure is undertaken in laminar flux hood using surgical sterile instruments. (Fig.10, Fig.11).



Fig.10. Laminar flux hood



Fig.11. Microsurgical instruments used in surgical experiments

The iodine solution is applied on the head of the mouse to avoid infections. The skin and calvaria of the skull are quickly removed in order to expose the cerebrum (Fig.12).



Fig.12. The cerebrum entirely exposed

Using a rubber spatula and a microdissector the cerebrum is removed from the skull and immersed in cold PBS (phosphate buffered solution) (2-4 degrees C) (Fig.13)

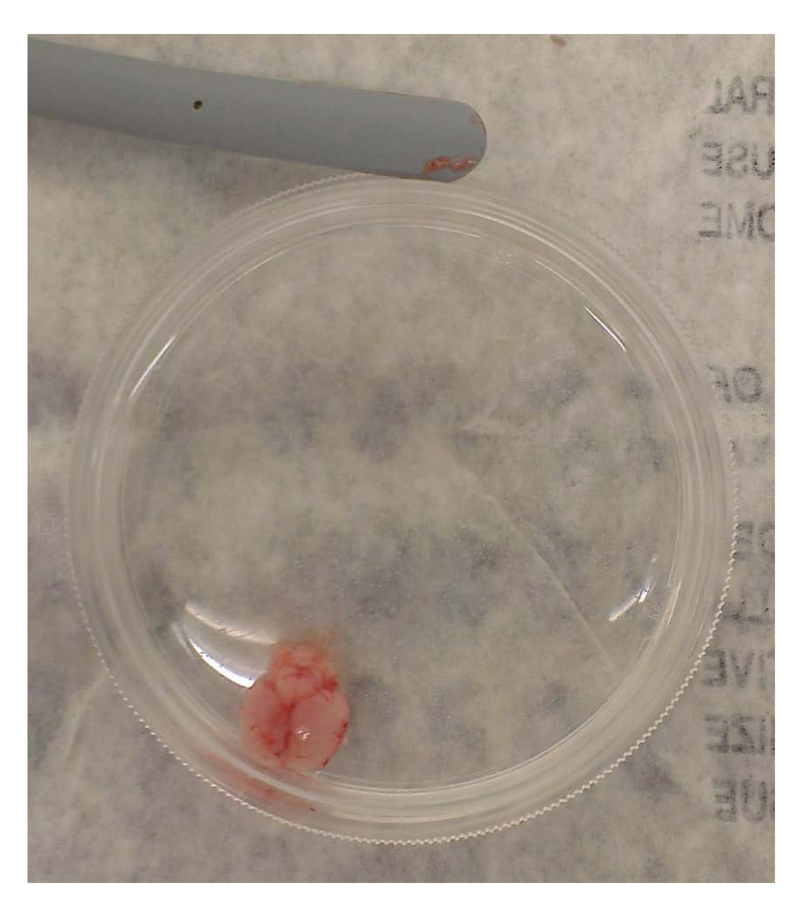


Fig.13. Isolated cerebrum immersed in cold PBS

The cerebrum is cut in thin slices of approximately 500 micrometers using a microtome (Fig. 14). These sections are cultivated on insert plates with 0,4 micrometer diameter (Fig.15). This special insert plate maintain the viability of the cerebral tissue sections.



Fig.14. Microtome for tissue sections.

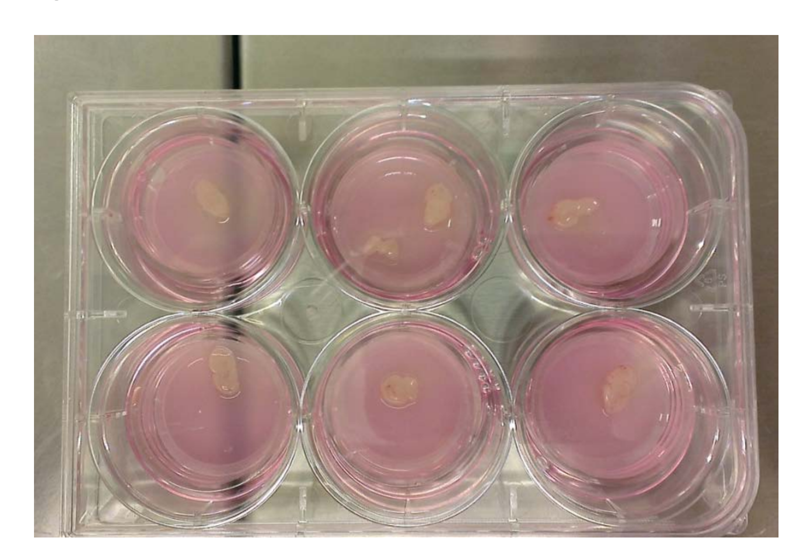


Fig.15. Tissue sections placed on culture membranes.

We used DMEM medium enriched with glutamina, fetal ser 15% and antibiotics 1% to cultivate the sections. After 48 h, we initiated the inoculation of GFP-marked glioblastoma cells. We used HTB14 (U87) glioblastoma cells, transfected with GFP "green fluorescence protein" gene(Fig.16). In cadrul proiectului am avut la dispozitie doua linii celulare: linia U87 si linia U251. Prin cultivari succesive, am constatat ca linia U87 isi mentine ritmul constant de crestere, iar experimentele ce implica aceasta linie

sunt reproductibile. Prin urmare am optat pentru utilizarea liniei HTB14 (U87) pentru experimentele din cadrul etapei II.

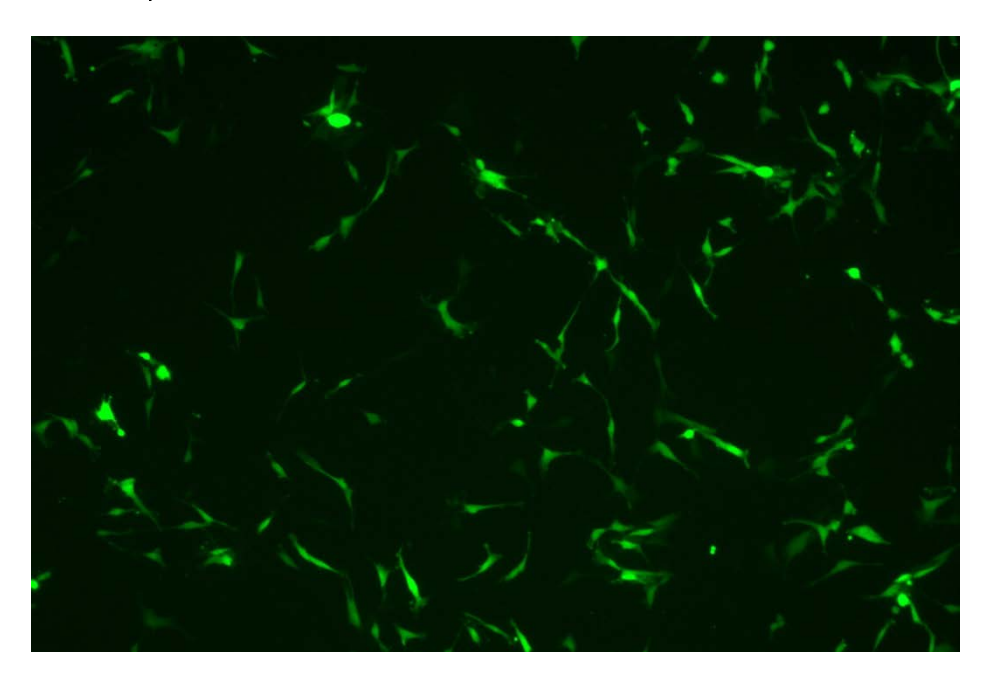


Fig.16.The HTB14 (U87) line transfected with GFP gene

The cells are inoculated as cellular suspension. Therefore, the glioblastoma cells will be detached from the cell culture using tripsine. Weconcentrate the cells at 10.000 cells per micro liter. For cerebral tissue section inoculation, we used an automatic injector with a Hamilton syringe. (Fig.17). We usually inject 5 micro liters, which contain 50.000 de cells.

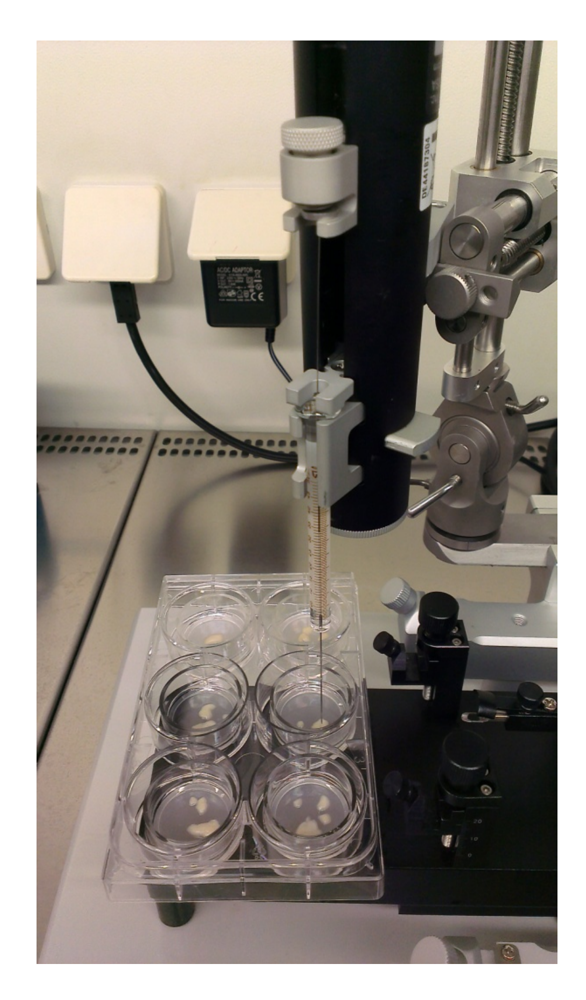


Fig.17.Automatic injector with Hamilton syringe

After cellular injection, the migration of the glioblastoma cells is monitoring by digital images registration using the Zeiss Axiovert A1 microscope at 1, 2, 3, 5, 7, 9, 12 days after cellular inoculation (Fig. 18).



Fig.18. Axiovert A1 Zeiss microscope with fluorescent filters and image acquisition system.

By strictly following the literature protocol, we noticed that many of tumoral cells spill over the edges of the section (Fig.19).

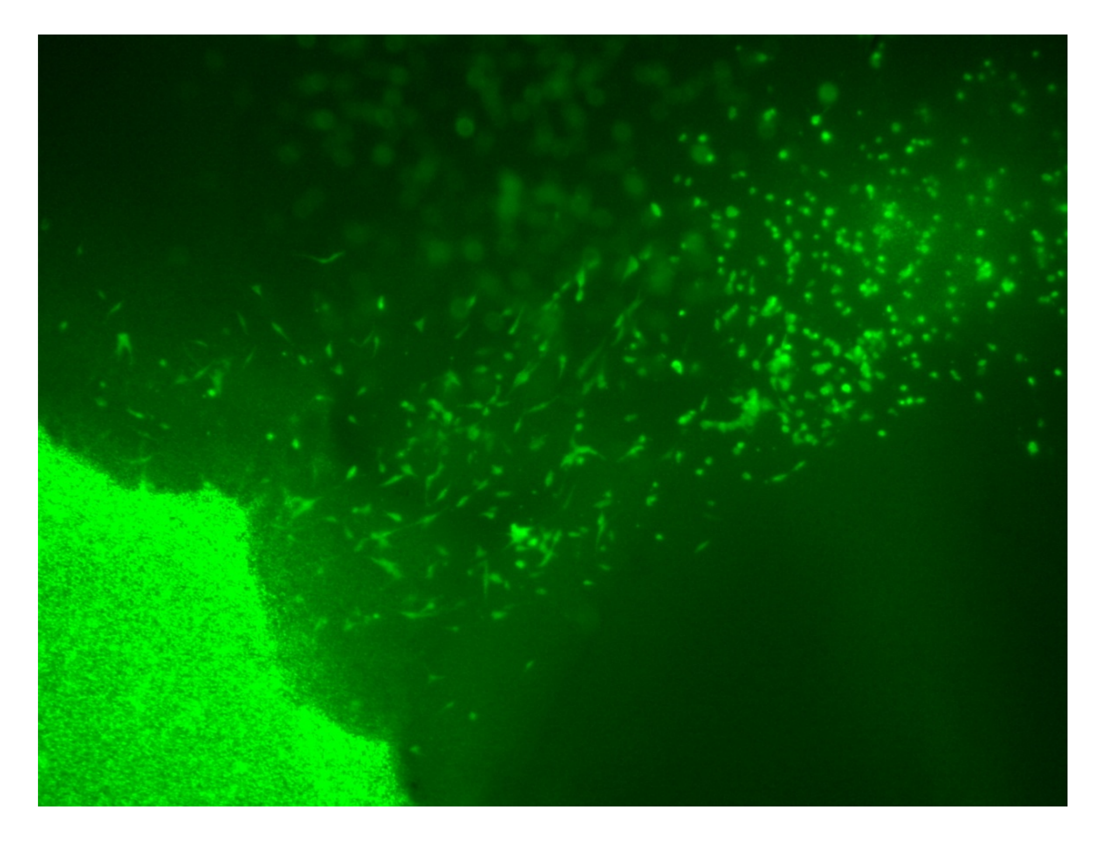


Fig. 19. Inefficiency of cellular inoculation inside the cerebral tissue sections due to the particularity of the shape of the Hamilton syringe.

The explanation of this phenomenon is the shape of the tip of the Hamilton syringe. Therefore we adapt the protocol of cellular inoculation. We cut perpendicularly the tip of the Hamilton syringe, thus the tip of the syringe became entirely included in the depth of the tissue section and the majority of the tumoral cells remained included in the section (Fig. 20).

We used the soft AxioVision SE64 Rel. 4.9.1 to measure the distances of cells migration. The time of inoculation was set up as reference point: t0. The images took immediately after inoculation revealed the presence of a round fluorescence cells at the inoculation site (Fig.20)

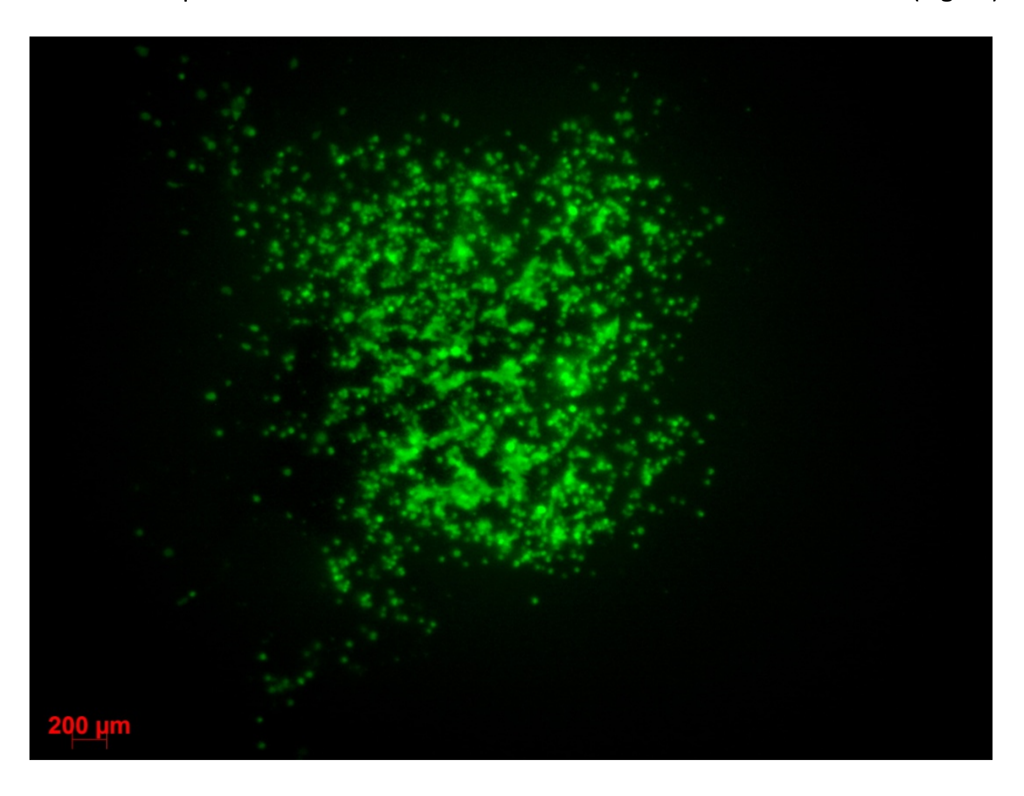


Fig.20. The phenotypic aspect of the glioblasotma cells HTB14 transfected with GFP, at the inoculation site

At 24 after inoculation, even if there are some migratory cells, the majority of cells preserve the initial aspect (Fig.21).

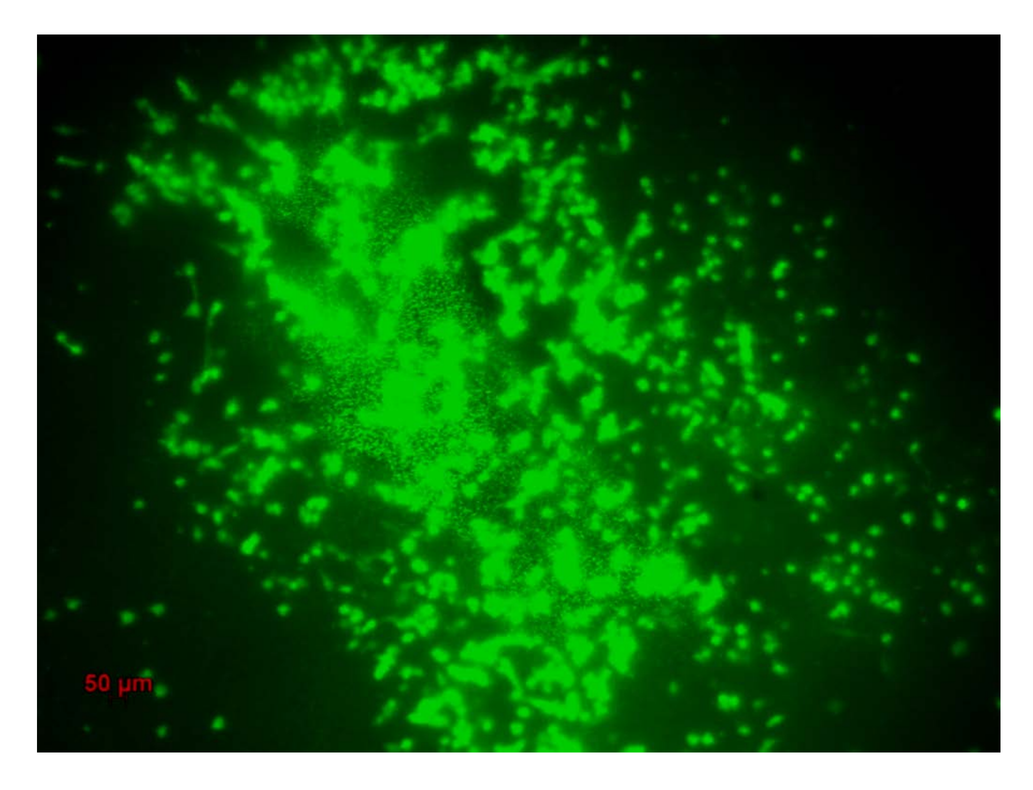


Fig.21. The shape of the glioblastoma GFP-HTB14 cells at 24 hours after inoculation

At 72 hours after inoculation, many tumoral cells change the phenotypic aspect and start to migrate to the periphery of the (Fig.22)

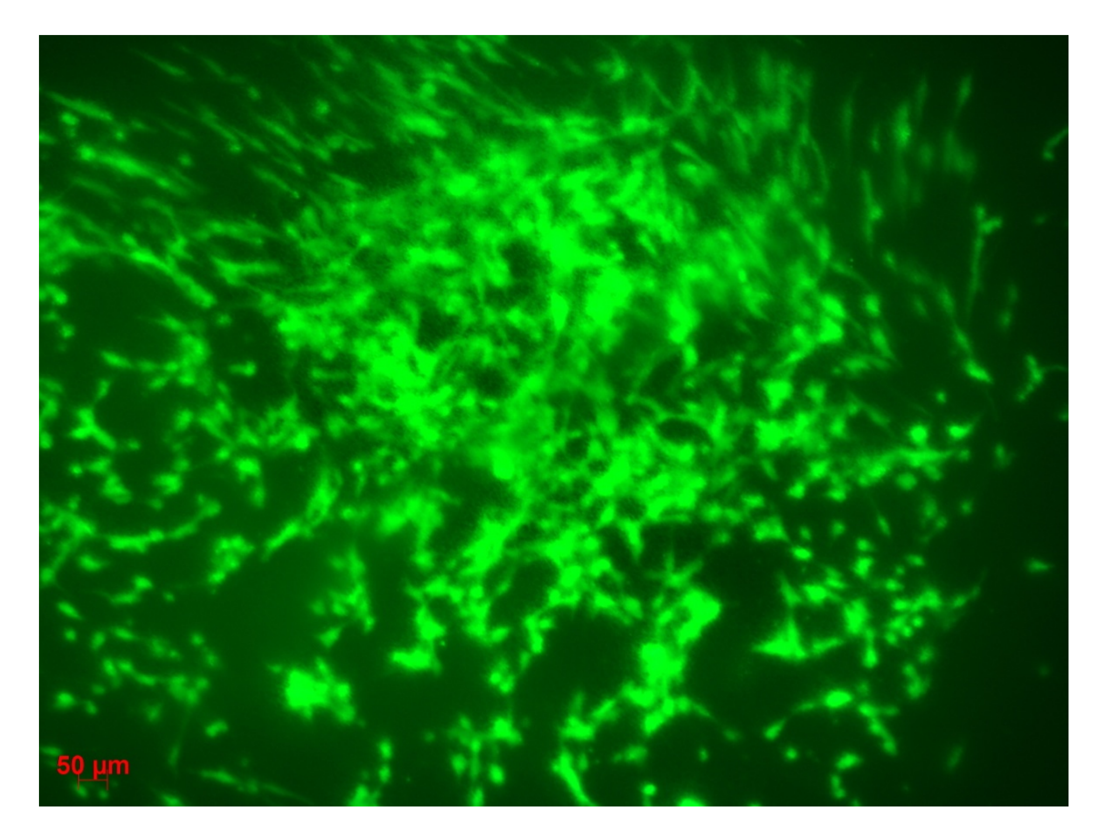


Fig.22. Glioblastom GFP-HTB14 cells at 72 hours after inoculation. You can notice the infiltrative phenotype at the periphery of the cellular population.

Using a great magnification (x20) we can notice the elongated shape of the infiltrative tumoral cell (Fig.23)



Fig.23. The characteristic shape of the infiltrative GFP tumoral cell

At 7 days after inoculation, the proliferation and the migration of the glioblastoma cells are evident, the tumoral cells invade almost entire tissue section (Fig.24).

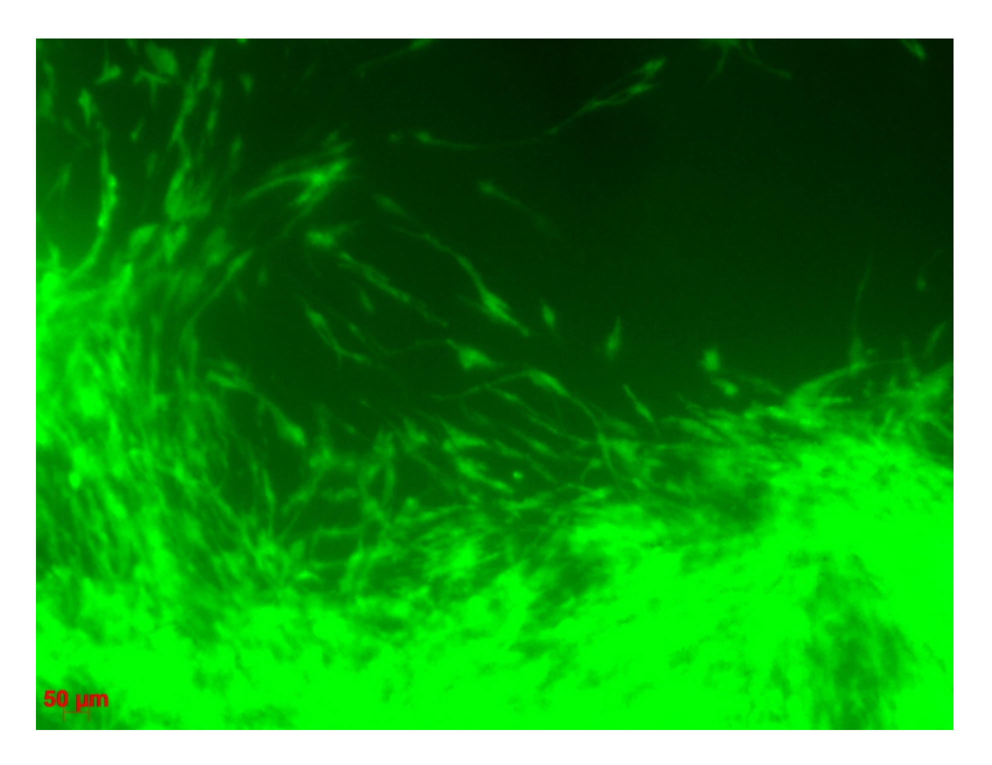


Fig.24. Glioblastom GFP-HTB14 at 7 days after inoculation

Modifying the protocols taken from the literature, we managed to develop a better experimental model of glioblastoma invasion which allowed us to visualize and monitor the invasion of glioblastoma cells in tissue.

Objective II. Gene/Molecule blocking, involved in glioma invasion – evaluation through "scrape migration assay" technique ":

Activity 2.1 Gene/Molecule blocking in glioblastoma cell-lines and primary glioblastoma cultures.

This activity has been achieved by the subcontractor This institute of Cellular and Molecular Pathology " Nicolae Simionescu" and it had the following experiments: 1. Obtaining the HTB-14 which super-expressed the GFP gene (HTB-14-GFP) and 2. Obtaining cellular lines HTB-14 which over-express GFP and don't express LIS1 (HTB-14-GFP-shLis).

1. Obtaining the celles from the HTB-14 which overexpress GFP. Obtaining this genetically modified cellular line was achieved through transfection of the HTB-14 cells with plasmide which contain the GFP gene. HTB-14 were cultivated in DMEM medium with 4.5% glucose, bovine fetum serum, antibiotic (peniciline, streptomicine, neomicine). In order to transfect, the cells have been passed through tripsinization in plastic clicks of 5cm in diameter with a density of 105 cells/cm2. The plasmids wich codify the gene for GPF (Fig. 25) were purchased from Clontech, amplified in bacetrias DH5, purified using a midiprep kit from Quiagen. The DNA obtained was

quantified through spectophotometry and were diluted until a concentration of 1mg/ml was obtained.

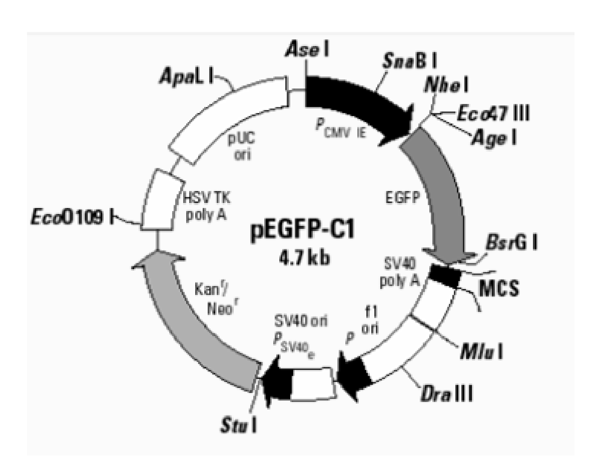


Fig25. The plasmide which codified GFP

Cellular transfection has been achieved through lipofection, using lipofetamine (Invitrogen), using the protocol of the provider. Thus, complexe DNA liposomes were prepared using 5ug DNA and 20uL Lipofectamine, in a volume of 500uL Optimem(Invitrogen). After 48h from trasnfection, the medium has been changed with a fresh one. Then, over a period of 30 days, cells have been cultivated in raising concentrations of geneticin 418 (G418), for the selection of transfected cells. In short, the medium has been changed twice a week, with a concentration of G418 starting from 400 and reaching 1000 ug/ml. finally some stable lines have been obtained (HTB14-GFP), out of which we have selected the line that expressed the strongest GFP, as we can observe in Fig.26.

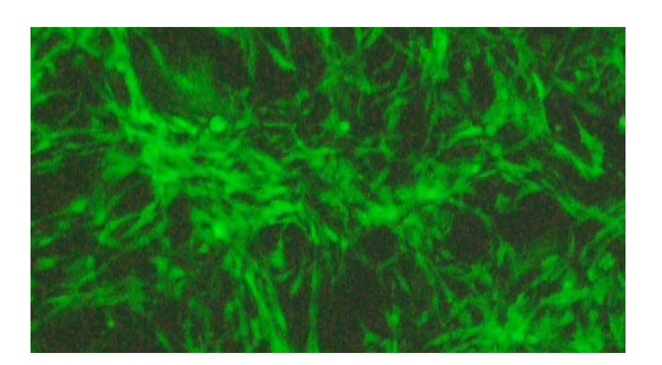


Fig. 26 Optical fluorescent microscopy used for cellular visualization HTB-14 which express GFP. We can observe the cellular distribution of the green fluorescent protein.

Obtaining the cells from the HTB-14 line which over-express the reporter gene GFP and did not express the LIS1(HTB-14-GFP-shLIS). In order to obtain the cellular line HTB-14-GFP-shLIS1, the HTB-14-GFL cells have been trasfected with plasmids which tonaint shRNA for LIS1 gene. HTB-14-GFP cells have been cultivated in DMEM medium with 4.5% glucose, suplimented with fetal bovin serum (10%) and antibiotic (peniciline, streptomicine, neomicine). In order to transfect the cells with the plasmids, the cells have been passed through tripsinization in plastic clicks of 5 cm in diameter and a density of 105 cells/cm2. For the trasnfection, the cells have been cultivated in DMEM buffered with HEPES and sodium carbonate and suplimented with hipoxantine, timidine, sodium private, L-glutamine, oligoelements, growth factors; in the culture medium for the transfection the protein level is at a minimum – insulin and transferin being the only proteical supplements.(SantaCruz-Plasmid Transfection Medium sc-108062). The plasmids used for the inhibition of the LIS1 gene contain 3 types of specific shRNA for LIS1, cloned in a lentiviral vector. Each shRNA sequence contain 19-25 nucoletides that have a "hair-needle " structure – which leads to the blocking of the gene in discussion.Plasmids with shLIS contain a gene resistant to puromicine for the selection of the cells that express stable shRNA. Transfection has been achieved with Plasmid Transfection Reagent sc-108061 reactive, from Santa Cruz, using the provider protocol. Thus, 2ug of shLIS plasmide has been dissolved in 200ul(final volume) medium sc-108062, and the trasnfection reactive (1-6ug), was diluted in 200 ul(final volume) medium sc-108062. The obtained mix between the plasmide solution and the trasfection reactive was added on the HTB-14-GFP cells that were washed and cultivated in sc-108062 medium. After 48h after trasnfection the medium has been changed and puromicine has been added in a final concentration of 0.5-2ug/ml.Puromicine is an antibiotic which inhibits proteical synthesis in cells that have not been trasnfected and do not have the puromycin N-acetyl-transferaze gene – which offers them reistence. Cells that were resistant have been selected. Testing through Real Time using specific probes show a drastical diminishing of the LIS1 genes in the cells trasnfected with shLIS.

Activity 2.2. Evaluation of inhibition of invasion assessed by „scrape migration assay" technique (surface invasion).

For this activity we used the HTB14 (U87) cells transfected with GFP- shLIS1. These cells have a decrease activity of LIS1 gene. As control we used same cells transfected only with GFP gene. We obtained both tyoe of cells (shLIS1-GFP and GFP cells). Both were cultivated at a density of 1 milion cells/ml. Cultivation was in DMEM medium enriched with glutamin, fetal seruml 15% and antibiotic 1%., at 37 degree C and CO2 5%. After 48 hours we performed the "scrape migration assay" test, a simple test which evaluate the migration of cells on a surface. We scrape the surface of the dish with a sterile instrument of 3 mm width and we take the image of the dish at t0 and at 1,2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 hours. We compare the images and analyze the time of proliferation and migration of tumoral cells which repopulate the scraped area of the culture dish. We present below the images taken at t0, 12 and 24 hours for GFP cells and shLIS1-GFP cells. The images were taken by optic microscope in phase-contrast at X5 (Fig.28).

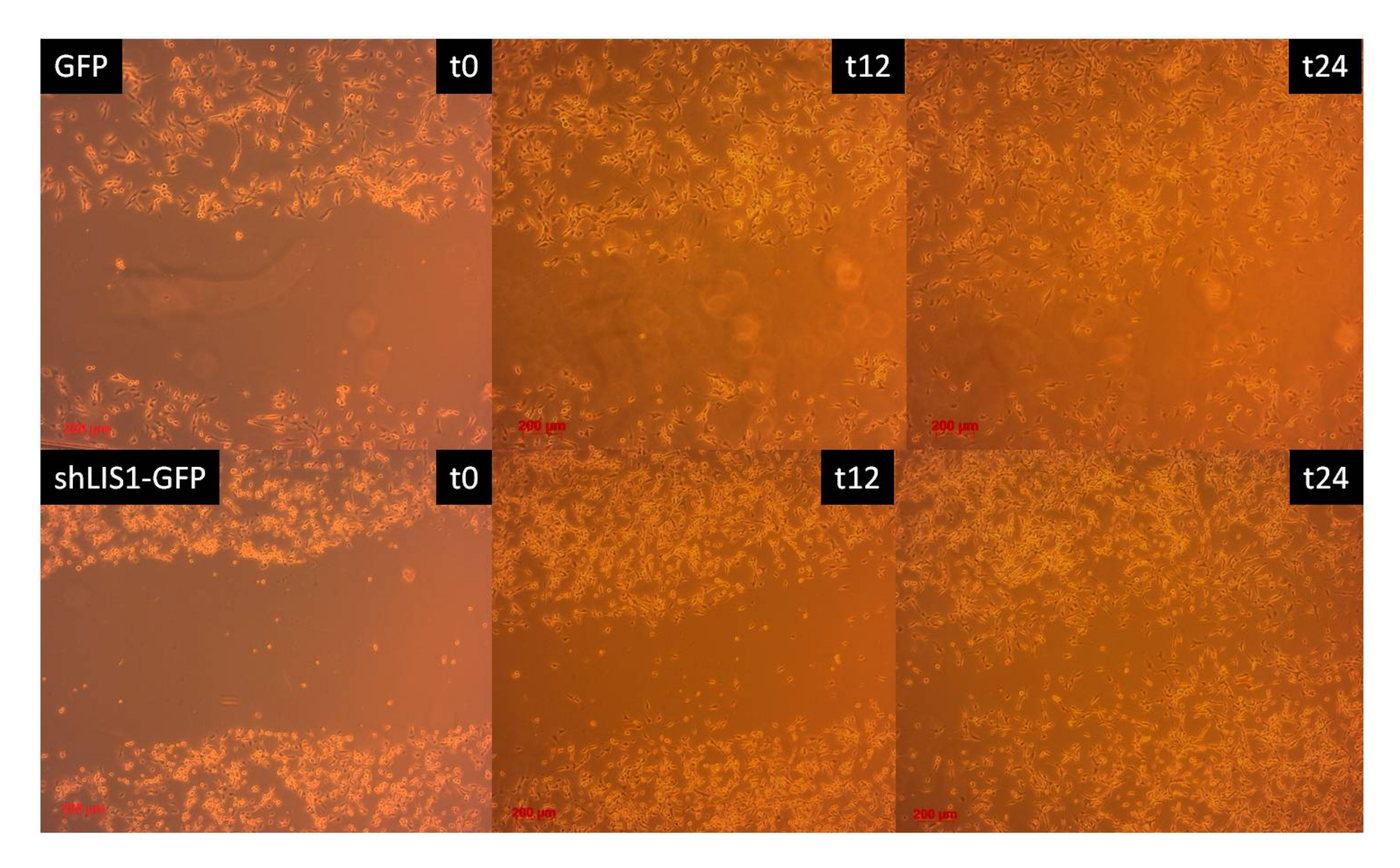


Fig.28. "Scrape migration assay" test for GFP-HTB14 GFP (top) and for shLIS1-GFP HTB14cells (bellow) at three key points: t0, 12 and 24 hours.

The preliminary analyzes of the images suggests that shLIS1-GFP cells have an advantage of proliferation and migration compared with GFP-cells. It can be observed that shLIS1-GFP cells almost entirely repopulate the scrape area at 24 hours in contrast with control cells, were the scrape are remain visible at 24 hours. The experiments will continue during the third phase of the project in order to confirm and validate these preliminary results.

Disemination of the results

The results of the projects were published in the following papers:

1. Immunohistochemical analysis of GFAP-δ and nestin in cerebral astrocytomas. Brehar FM, Arsene D, Brinduse LA, Gorgan MR, BRAIN TUMOR PATHOL. 2014 Sep 2. [Epub ahead of print], DOI 10.1007/s10014-014-0199-8 (ISI, Impact factor2013: 2,281)

2. GFAP-δ and Nestin as Molecular Markers related to the Cell Origins and Invasion in Human Gliomas, F. M. Brehar, M. R. Gorgan, oral presentation at the 3rd Congress in the Danube-Carpathian Region Joint Meeting with Southeast European Neurosurgical Society(SeENS). Abstract published in J NEUROL SURG A CENT EUR NEUROSURG 2014; 75 - o009, DOI: 10.1055/s-0034-1382170 (ISI, impact factor2013: 0.493)

3. Nestin expression in biopsy samples correlates with the invasive phenotype of cerebral gliomas. F. M. Brehar, D. Arsene, M. Lisievici, M. R. Gorgan. Oral presentation. 9th CONGRESS of the RSN with International Participation, September 19-21, 2013, Bucharest, Romania.

4. Glioma stem cells specifically induce infiltrative growth pattern xenografts. F. M. Brehar, R.M. Gorgan, C. Bleotu, O. Zarnescu. Poster presentation. EANS Annual Meeting 2013, November 11-14 2013, Tel Aviv, Israel.

C. Activities developed during the third part of the project (2015)

Objective 1. Inhibition of genes/molecules invovlved in gliomas invasion –„transwell migration assay" technique:

Objective 2. Inhibition of genes/molecules invovlved in gliomas invasion –„in tissue"technique: –

- Activity 2.2. Inoculation of invasive down-regulated glioblastoma primary cultures in cerebral sections

- Activity 2.3. Evaluation of innasion in the new invasive model

Activity 2.2. Inoculation of invasive down-regulated glioblastoma primary cultures in cerebral sections

As a part of this activity we improved the technique of inoculation of the mice cerebral sections with GFP-glioblastoma cells. We performed 7 experiments. For each experiment we used 3 mice. We improved the following technical aspects.

1. In order to prolong the viability of the cerebral sections we used for the experiments younger mice, of no more than six day-old. In these cases, the cerebral metabolism is set-up for anaerobe function, and it is able to survive in vitro for longer periods. We used the same protocol as in the previous stage, to induce the barbituric coma and profound hypothermia before removing the mice brain. The experiments were conducted according with the national and European legislation and with the approval of the Ethic Committee of "Bagdasar-Arseni" Clinical Hospital. The mice brain is immediately introduced in cold PBS solution (Fig.39a), than is mounted in the microtome stage (Fig.39b).

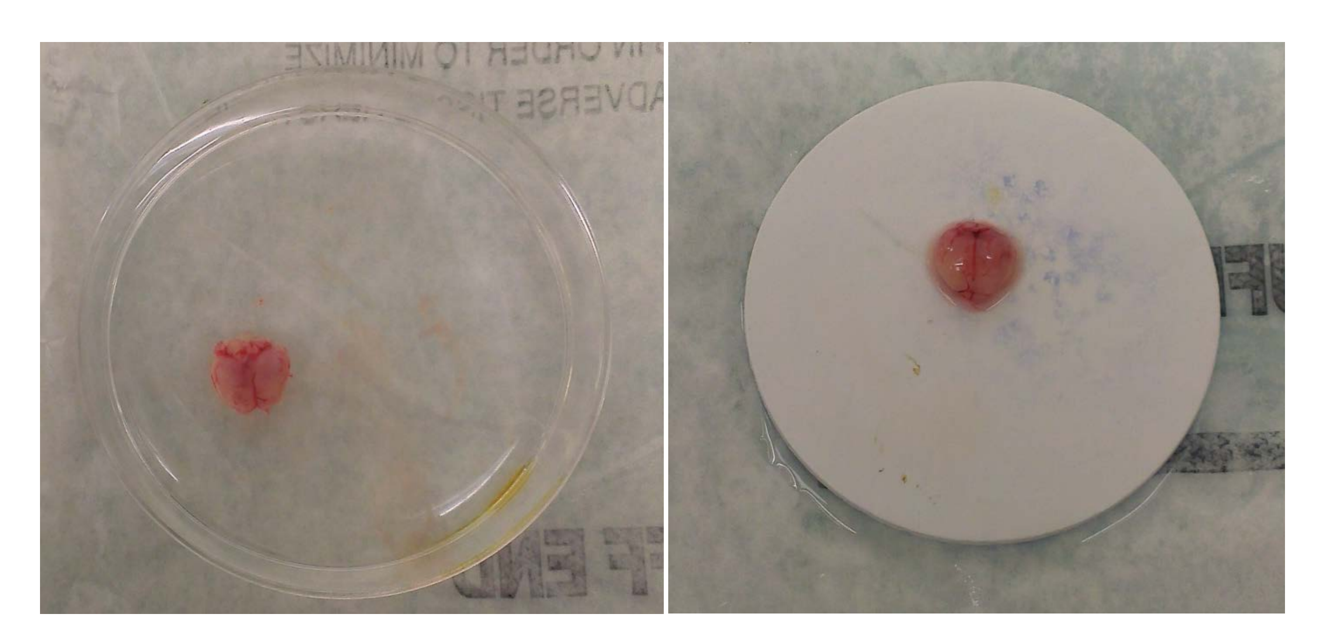


Fig.39. The macroscopic aspect of the mouse brain, a. immersed in cold PBS and b. mounted on the microtome stage.

The cerebral sections are than cultivated on milipore membranes inserts introduced in a six-well plate (Fig.40). For cultivation we used DMEM medium enriched with 20% fetal serum, glutamin 1% and penicilina-streptomicina 1%. Another important aspect is the quantity of the medium used. Medium should not cover completely the cerebral section, but rather, it should be placed at the medium-air interface. Proceeding in this way, there will be enough medium to reach the section, but there will be not too much medium which can detaches the section from the membrane. We reached to the conclusion that the quantity of the medium should be between 800 and 1000 micro-liters for each well. It is also very important to change daily the medium in order to maintain the viability of the cerebral section. The sections are then cultivated at 37 degree Celsius in humidified atmosphere enriched with 5% CO_2.

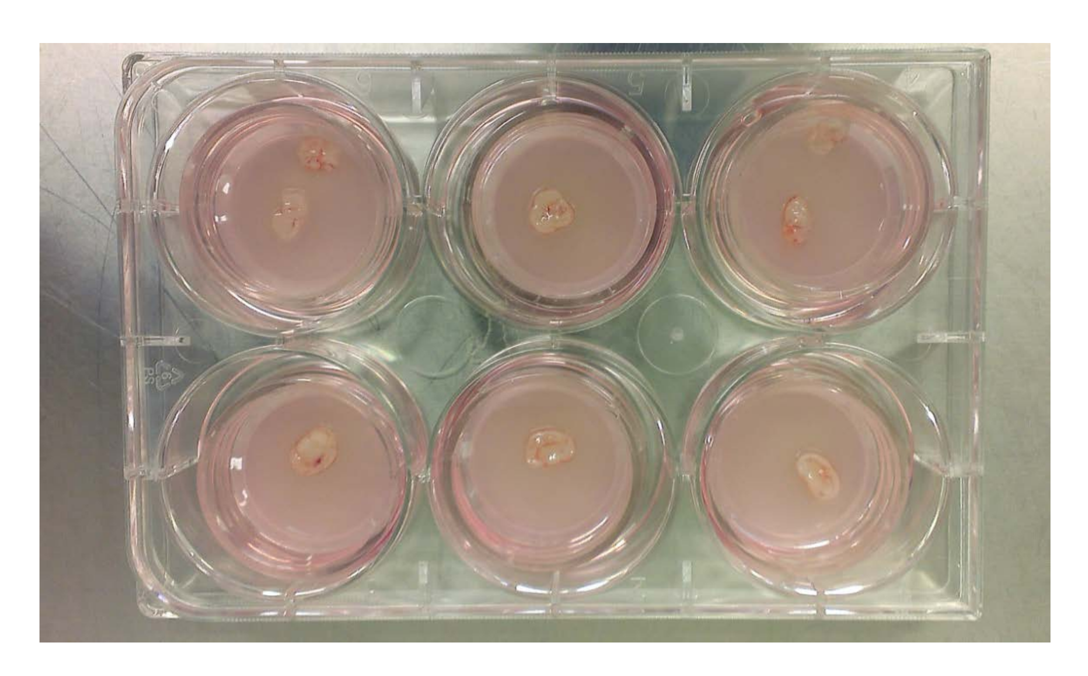


Fig.40. Cerebral sections cultivated on porous membranes placed on the insets introduced in a six-wells plate.

After 48-72 hours of cultivation, the cellular suspension can be inoculated in the cerebral sections. Another pitfall, frequently mentioned in the literature, is represented by the difficulty of injecting strictly inside the width of the section of the cellular suspension. We found in many experiments that the majority of the cells are located on the periphery of the section and not inside the section, because of the leakage of the cellular suspension during inoculation procedure (Fig.41).

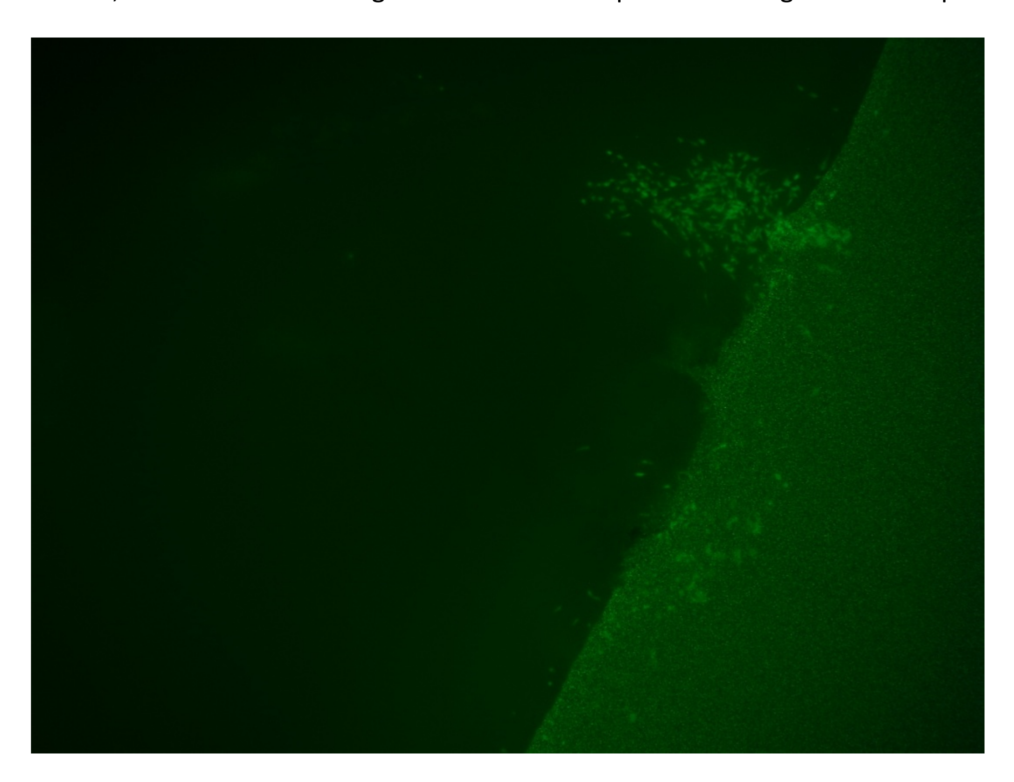


Fig.41. The periphery of a cerebral section immediately after the inoculation of fluorescence labeled HTB1 cells. The majority of the cells are located at the periphery of the section.

In order to avoid this pitfall, the authors imagined another technique of cellular inoculation, which used for cells inoculation a 10 micro-liters micropipette instead of the Hamilton syringe (Fig.42). This new technique improve the rate of success of the inoculation procedure.

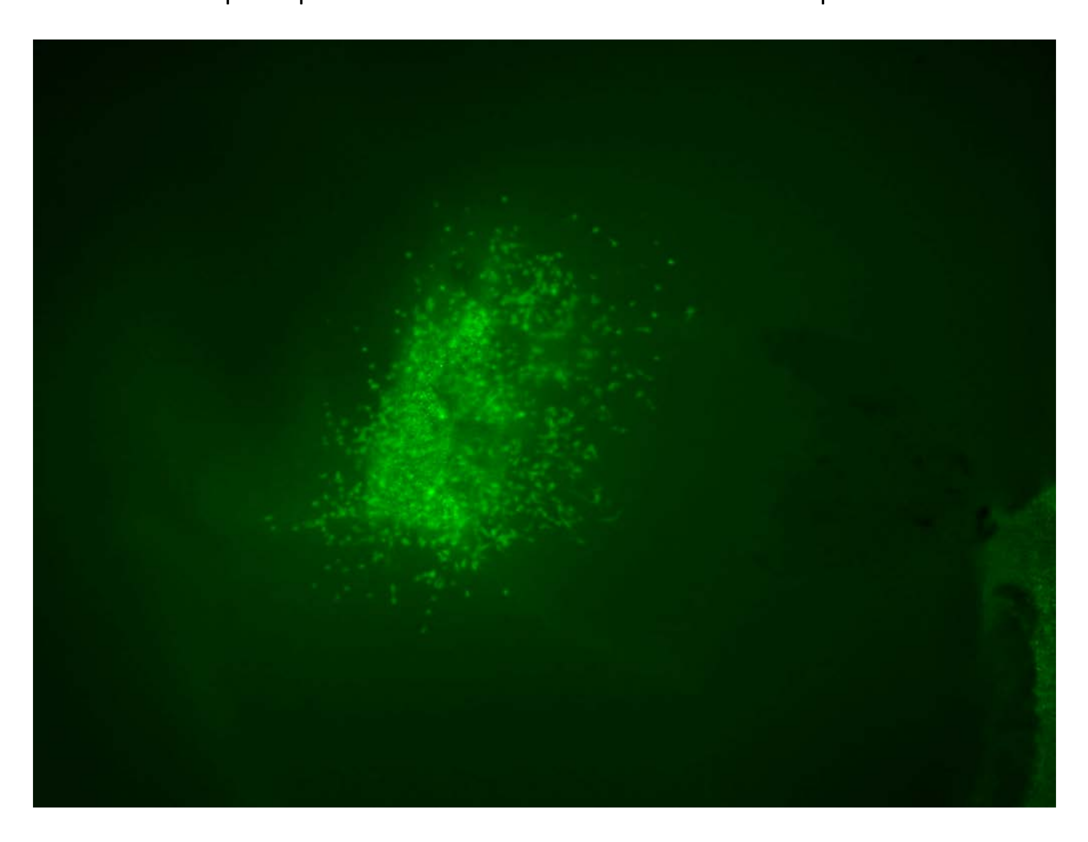


Fig.42. The fluorescence image of the cerebral section after inoculation of the fluorescence labeled HTB14 cells. Cells are located at the central portion of the section.

This experimental model was validated by testing it in different experiments. The HTB14 cells were able to invade progressively the cerebral section in 11days after inoculation. In other experiments, the HTB14 cells were faster, being able to infiltrate the cerebral section in only 7 days after inoculation (Fig. 43a,b).

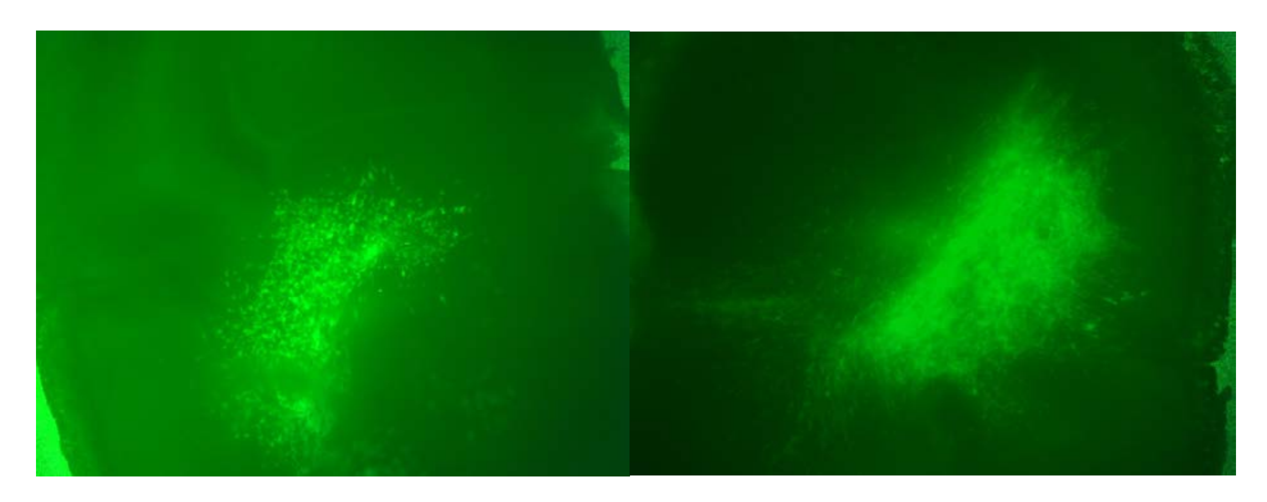


Fig.43.Cerebral section at a. 24 hours after HTB14 cells inoculation and at b.11 days after inoculation.

In the other experimental series we found a high invasive capacity of the glioblastoma cells, with a complete infiltration of the cerebral section within 7 days after inoculation. (Fig.44)

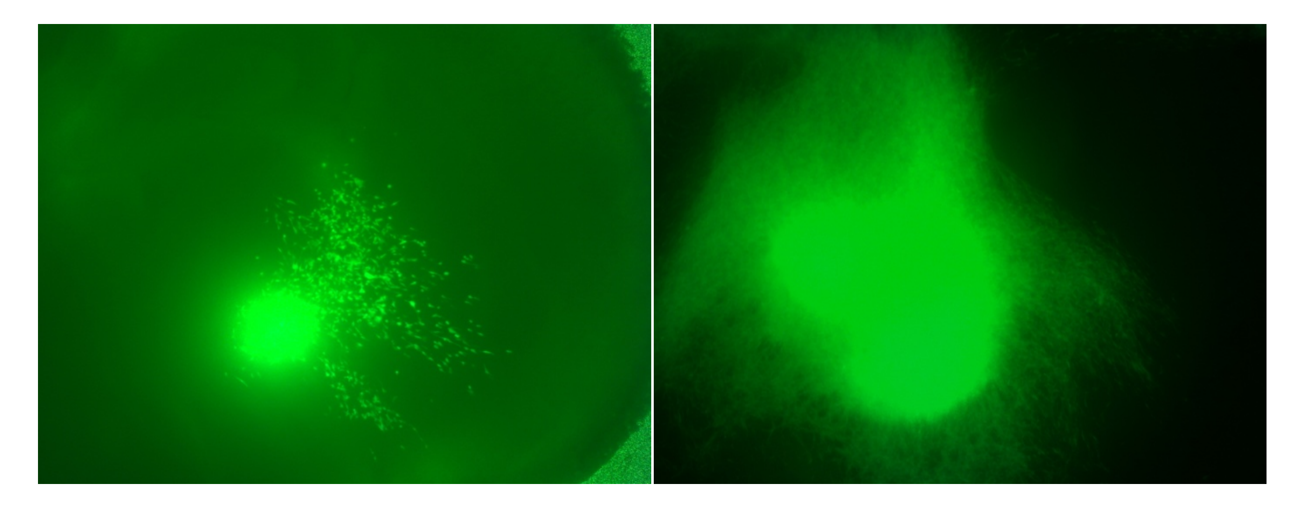


Fig.44 a Cerebral sections at a. 24 hours and b. 7 days after HTB14 cells inoculation.

Using a greater magnification (x20), we can easily observe the particular phenotypic aspect of the infiltrative glioblastoma cells, respectively the elongate shape, with one or two prolongations at the extremities of the cells (Fig.45).

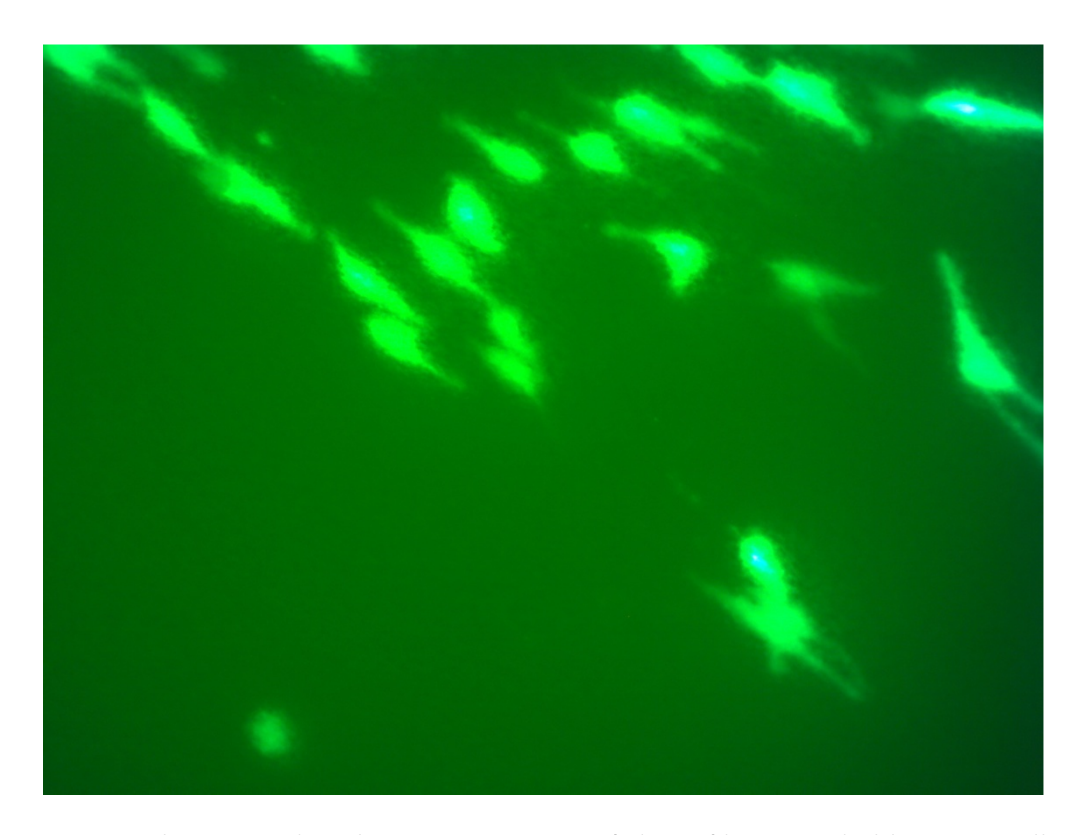


Fig. 45. The particular phenotypic aspect of the infiltrative glioblastoma cells, respectively the elongate shape, with one or two prolongations at the extremities of the cells

Results:

1. Nestin expression in biopsy samples correlates with the invasive phenotype of cerebral gliomas. F. M. Brehar, D. Arsene, M. Lisievici, M. R. Gorgan. Prezentare orala. 9th CONGRESS of the RSN with International Participation, 19-21 Septembrie, 2013, Bucuresti, Romania.

2. Glioma stem cells specifically induce infiltrative growth pattern xenografts. F. M. Brehar, R.M. Gorgan, C. Bleotu, O. Zarnescu. Prezentare poster. EANS Annual Meeting 2013, 11-14 Noiembrie 2013, Tel Aviv, Israel.

3. GFAP-δ and Nestin as Molecular Markers related to the Cell Origins and Invasion in Human Gliomas, F. M. Brehar, M. R. Gorgan, prezentare orala la 3rd Congress in the Danube-Carpathian Region Joint Meeting with Southeast European Neurosurgical Society(SeENS). Abstract publicat in J NEUROL SURG A CENT EUR NEUROSURG 2014; 75 - o009, DOI: 10.1055/s-0034-1382170 (Revista indexata ISI, factor de impact 2013: 0.493).

4. Immunohistochemical analysis of GFAP-δ and nestin in cerebral astrocytomas. Brehar FM, Arsene D, Brinduse LA, Gorgan MR, Articol in extenso publicat in Brain Tumor Pathol. 2015 Apr;32(2):90-8 (revista indexata ISI, factor de impact 2014: 1,23).

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Date: 03.12.2015

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